PRESIDENCY UNIVERSITY

DEPARTMENT OF LIFE SCIENCES

PhD Thesis Synopsis

Title: **Characterization of Indigenous Sewage Microbial Communities of Kolkata and their Bioremediation Potentiality in Heavy Metal Contaminated Soil**

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Registration Number with date: **R-14RS18210015 (Date: 5th May, 2016)**

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I. Abstract:

Culturable bacterial profile, their chromium tolerance and removal were investigated in this study. The literature survey revealed that the microbes growing in heavy metal polluted soil can furnish an impressive mode of environmental heavy metal degeneration. In order to understand the activity of microbial heavy metal tolerance, rhizospheric microbes were isolated from the soil of three hot and humid, heavy metal depository sewage reservoirs in the south (Circular Canal, Tolly Nullah), east (East Kolkata Wetland), and north (Kestopur Khal) of Kolkata. Different morphological, physiological, and biochemical characteristics and antibiotic resistance patterns were analyzed with these isolated microbes. In order to compare the composition of the bacterial community, the selected microbes were subjected to molecular characterization. Their heavy metal resistance patterns and possible biochemical mode of action in reducing the heavy metal concentration was subsequently estimated. The Plant Growth Promoting Rhizobacteria (PGPR)-like traits of the isolated soil microbes were investigated and their influence on rhizospheric heavy metal absorption by the plant roots was studied. Three neutrophilic mesophilic chromium resistant microbial strains (*Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018) were chosen for their selective antibiotic sensitivity and effective chromium absorption and reduction capability, which are characteristics of a good bioremediating candidate. They may help to protect humans from heavy metal toxicity by reducing soil metal accumulation and also reduce heavy metal absorbance of edible plants.

II. Introduction:

The modern-day industrialization and urbanization have led to the deposition of heavy metals in the soil, water, and the atmosphere. This pollution seriously affects the lungs, kidney, cardiovascular and central nervous systems and even leads to cancer. Accumulation of high concentration of heavy metals also affects plant growth and metabolism adversely.

Bioremediation is a suitable method to combat the problem which helps degrade, remove, or utilize organic contaminants like heavy metals, oils, and other substances from waste by the activity of selected plants or microorganisms. The microbes growing in urbanized sewage sites can resist heavy metal toxicity and detoxify soil by developing different specialized mechanisms including efflux, accumulation, heavy metal reduction, utilization of metal ions in physiological processes, for instance as terminal electron acceptors during respiration, or acid production [Nageswaran *et al.* 2012; Valverde *et al.* 2011] to remove heavy metals from the environment. Many prior reports have demonstrated the role of waste land resident soil microbes in metal abolition [Kelly *et al.* 1998; Vargas-Garcia *et al.* 2010; Kamika and Momba 2013; Gupta *et al.* 2014]. Different species of *Bacillus, Staphylococcus, Pseudomonus, Microbacterium, Micrococcus, Mangrovibacter yixingensis, Klebsiella, Desulfovibrio and Escherichia coli, Agrobacterium radiobactor, Aeromonus dichromatica* participate in metal bioremediation and most of them exhibit reduction of hexavalent chromium (Cr^{6+}) to less toxic trivalent chromium (Cr^{3+}) [Sanjay *et al*, 2018; Wang and Shen,1995; Upadhayay *et al*, 2017; Kaushik *et al*, 2012]. Previous findings also demonstrated that soil microbes can accumulate metals within their cells at a concentration 50 times higher than that of their habitat [Pumpel et al. 2001].

Although the soil is the natural habitat of millions of soil-borne microbes, contamination with different hazardous pollutants reduces the concentration of soil microbes. However, the microbes in the plant rhizospheres can withstand polluted environments, since a considerable amount of soil microbes can survive in the heavy metal contaminated soil of the plant rhizospheres [Whipps 1990; Bais *et al.* 2006; Badri and Vivanco 2009; Badri *et al.* 2009]. So, if heavy metal degrading bacteria can aid plant growth and reduce the levels of accumulated heavy metals in plants, they can be implemented in poorly cultivated heavy metal contaminated environments [Nyamboya *et al.* 2013].

Previously, Ray Chaudhuri *et al.* (2012) reported that the hot and humid tropical climate of Kolkata aids the sewage microbial communities to thrive in the heavy metal contaminated environments of the different sewage canals. But there is no detailed report on the community composition study of heavy metal degrading microbes present in the different sewage regions of Kolkata. It is also unclear from previous studies whether the plant rhizospheric microbes can diminish plant heavy metal accumulation and promote plant growth. So, the significance of the present study lies in implying eco-friendly heavy metal remediating bacteria from the different sewage sites of Kolkata.

III. Objectives:

1. To analyze microbial bio-removal efficiencies against different heavy metals and attempt to determine their possible mode of action.
2. To check the diversity in community composition among bio-remediating microbes present in different wetlands of Kolkata and their molecular profiling.
3. To specify whether all rhizospheric microbes possess important role in decreasing plant heavy metal accumulation or not.

IV. Methodology:

1. Soil and plant Sample collection from selected sites:

Some edible herbs (*Amaranthus viridis, Coccinia cordifolia, Ipomea aquatica, Alternanthera sp., Colocasia esculenta)* and their rhizosphere soil (from 10 cm depth) samples were collected in triplicate from three selected sewage regions of south, east and north Kolkata, viz. Circular Canal (Tolly Nullah), East Kolkata Wetland and Kestopur Khal. The herbs were identified by Bengal Plants (Vol. I & II). The effluent discharges were also collected for Physico-chemical tests. The collected samples were stored in sterile pouch at 4^{o}C and Physico-chemical characterization was started within two hours of collection.

2. Physico-chemical profiling of soil:

After checking colour and texture, soil samples were dried. Dry soil was diluted five times in 0.01 (M) sterile $CaCl_2$ solution and pH was checked in electronic pH meter (GeNei).

The Chemical Oxygen Demand (COD) of the soil samples was measured by the open reflux method, in which mercuric sulfate, potassium dichromate, and concentrated sulfuric acid were added to 50% diluted sewage water, refluxed for two hours, and diluted to 150 ml. After cooling the sample, and titrated against 0.25 (N) FAS (ferrous ammonium sulphate) using Ferroin as the indicator. The end point of the titration was indicated by the keen change of color from blue-green to wine red.

The quantity of Dissolved Oxygen (DO) in the collected sewage sludge was measured by the Azide modification method, in which 300 ml of the freshly collected sample was mixed thoroughly with an equal volume of manganese sulfate and alkali iodide azide solution to obtain the precipitate. The settled precipitate was then dissolved in concentrated sulfuric acid and titrated against sodium thiosulphate using starch as an indicator.

In order to measure the soil dehydrogenase activity (DHA), 5 g soil was dissolved in 5 ml of 0.8% triphenyl tetrazolium chloride (TCC) and a few drops of 5% sodium sulfate was added to the mixture. Dissolved soil was centrifuged after one hour of dark shaking at 30°C, and the color intensity of the filtrate was measured by a spectrophotometer. The percentage of enzyme inhibition by the heavy metals was calculated from the standard curve prepared using a solution of tryphenyl formazan and ethyl alcohol [Weddle and Jenkins 1971]. The dehydrogenase activity

of the soil samples was measured by plotting the optical density (O.D.) values at 450 nm in the standard curve.

The normalized values of four different sewage physico-chemical parameters were used to correlate them with the microbial colony forming unit (CFU).

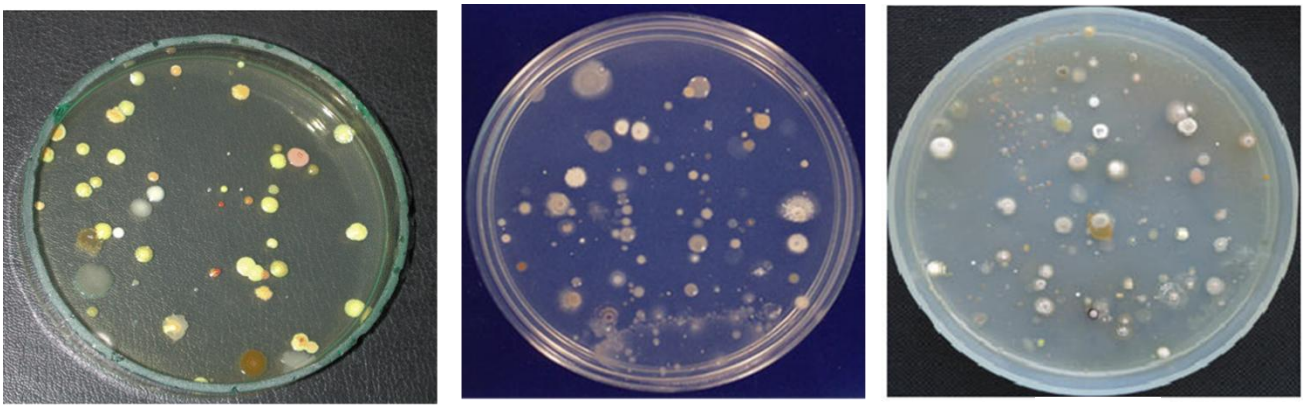
3. Metal estimation in soil, plant and bacterial sample:

The samples were properly lyophilized using a freeze drier and ground into fine particles. Approximately 150 mg (120 mg in case of bacterial pellet) of the finely ground homogeneous sample was placed on the face of the Pelletizer and the film on the pellet disc was removed. The pellets were inserted into the sample holder, attached with scotch tape, the heavy metal content was determined in triplicate by EDXRF (Energy Dispersive X-ray Fluorescence) spectrometer.

The pulverized soil, plant, bacterial pellet and culture media was also digested with HNO_3 and H_2SO_4 on hot plate for metal estimation by Atomic Absorption Spectrophotometer (APHA 23rd 3030 Ed.) model Variant AA140.

4. Isolation of soil bacteria:

One gram of each soil sample was dissolved in 10 ml of 0.85% NaCl containing sterile water and serially diluted. Then,1 ml from each dilution (10^{-1} to 10^{-11}) was swept on Luria Burtani agar plates and incubated at 37°C for 24 to 48 hours to obtain the number of colony forming unit (CFU) per gram of soil. The pure colonies, obtained from 10^{-7} times diluted soil suspension, were separated based on colony morphology and purified by repeated streaking. From the properly distinguished colonies, 54 microbial strains were differentiated by their colony morphology, streaked on the master plate and also preserved in 80% glycerol stock for further experiments.



[1A] **[1B]** **[1C]**

Figure 1: Bacterial isolates were obtained by spreading 10^{-7} times diluted soil samples collected from [A] East Kolkata Wetland, [B] Circular Canal and [C] Kestopur Khal

5. Spectrophotometric analysis:

The colour intensity and turbidity of different solutions were measured using visible spectrophotometer (Systronics 106) and UV-VIS spectrophotometer (Jasco V530) at required wave length.

6. Morphological identification of the soil microbes:

The cellular morphology of the isolated microbes was determined by the conventional Gram staining procedure (Bartholomew and Mittwer, 1952) in which the bacteria were stained with crystal violet and safranin (counter stain). The endospore-forming capability of the microbial isolates was studied by the Schaeffer-Fulton method where Malachite green was used as the primary stain.

7. Biochemical characterization of the soil microbes:

The different bacteria produced extracellular substances which were monitored using different commercially available supplementary media and subjected to different biochemical tests, described hereafter:

- **Gelatinase test:** Stab inoculates were prepared in nutrient gelatin tubes by puncturing agar columns. After freezing, the solidity of the medium was inspected.

- **Catalase test:** 1% H_2O_2 was applied on the inoculated culture tube and the appearance of effervescence indicated the presence of the catalase enzyme.
- **Amylase test:** 1% soluble starch supplemented nutrient agar plates were incubated at 28°C for 48 hours. A clear red zone around bacterial colonies after iodine application indicated starch hydrolysis. .
- **Fluorescence test:** Inoculated King's B medium plates were incubated at 28°C. After 48 hours of incubation, the plates were placed in a UV chamber for analyzing the fluorescence.
- **Methyl red Voges-Proskauer test:** A few drops of methyl red indicator were applied onto inoculated MRVP broth tubes and the change in color was observed after 48 hours incubation at 35°C.
- **Urease test:** Phenol red supplemented urea agar medium was inoculated and the change in color was observed after 48 hours of incubation.
- **Diffusible pigmentation test:** In order to detect pigmentation, inoculated plates containing King's B medium were observed after the first day and after six days of incubation.

8. Standardize the optimal microbial growth condition:

The bacterial isolates were grown under different initial pH (6.0, 6.4, 6.8, 7.0, 7.2, and 7.4) and different thermal environments (27°C, 37°C, and 47°C) in LB broth media for 24 hours to standardize their suitable growth condition. The efficiency of bacterial growth was assessed at 595 nm wavelength.

9. *Antibiotic screening*:

Four antibiotics viz. Ampicillin (10 µg/ml), Norfloxacin (10 µg/ml), Doxycycline (30 µg/ml), Tetracycline (30 µg/ml) were used to characterize microbial isolates. To measure bacterial resistance or sensitivity, antibiotic discs were applied to the Muller Hinton Agar supplemented with 10^{-2} times diluted log phase Muller Hinton suspension culture. The zone of inhibition against different antibiotics was standardized by the commercially available National Committee for Clinical Laboratory Standard's (NCCLS) chart.

10. Measure bacterial heavy metal resistance by the agar cup technique:

The resistance of isolated soil bacteria against trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) chromium, cadmium (Cd) and cobalt (Co) was measured individually and also in consortia implementing agar cup technique [Cooper 1955]. The salts of respective heavy metals were used at different concentrations (100mM, 50mM, 10mM, 10µM, 10 nM) to investigate and compare the inhibition zone. In this method, each inoculated plate was bored with sterile cork-borer (8 mm diameter) for creating wells of equal width and depth into which metal salt solutions were poured, and the zone of inhibition was subsequently measured.

11. Determination of MIC against chromium resistant bacteria:

For the determination of the Minimum Inhibitory Concentration (MIC), the microbes were inoculated in gradually increased concentrations of hexavalent chromium supplemented LB broth media till their growth was totally inhibited and bacterial MIC was determined by measuring turbidity at 595 nm [Yilmaz, 2003].

12. Evaluation of hexavalent chromium:

The presence of hexavalent chromium was confirmed by Diphenylcarbazile spectrophotometry method (APHA 23rd Ed.) in which the developed colour about 10 minutes incubated H_3PO_4 containing sample at pH 5 was measured at 350 nm (Sanchez-Hachair and Hofmann, 2018).

13. Detection and confirmation of trivalent chromium within cell:

The percentage of reduction of hexavalent chromium within bacterial cell pellet with relative growth of bacterial cell was assessed simultaneously as discussed earlier (Dey *et al*. 2014). Microbial growth stimulators like sodium azide and 2,4 dinitrophenol (DNP) were also applied at 10 mM Cr supplemented media with eqimolar concentrations to find the rate of microbial chromium reduction in the presence of these stimulators.

The presence of trivalent chromium within the bacterial cell pellet was confirmed by applying NaOH and NH_4OH. Gray green precipitate was formed in presence NaOH was readily soluble in excess alkali. But the precipitate formed in the presence of NH_4OH didn't dissolve by adding excess ammonia. Residual hexavalent chromium content was measured by Diphenylcarbazile method.

14. Molecular characterization by 16S rDNA sequencing:

The bacterial genomic DNA was isolated with the microbial DNA isolation kit (GCC Biotech) according to the manufacturer's protocol, and the 16S rRNA region was amplified using BDT v3.1 cycle sequencing kit and 27F forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse (5'-GGTTACCTTGTTACGACTT-3') primers. The purified PCR product was sequenced using ABI 3730Xl DNA analyzer executing Sanger sequencing method.

The 16S rDNA sequences were annotated using Chromas Lite software (version 2.01), and their near relatives and taxonomic position were identified by the NCBI MEGABLAST algorithm [Altschul et al. 1990; Zhang et al. 2000]. Multiple sequence alignments, phylogenetic analyses and molecular evolutionary studies of the 16S sequences were performed using the Clustal Omega [Maderia *et al*. 2019] and MEGA10 [Tamura *et al*. 2013; Kumar *et al*. 2018; Kimura 1980]. The partial 16S rDNA sequences acquired herein were submitted to the NCBI GenBank database, and their accession numbers were subsequently obtained.

15. Profiling bacterial growth in the presence and absence of heavy metals:

The hourly bacterial growth pattern was monitored in trivalent and hexavalent chromium supplemented and heavy metal free LB broth to obtain the growth curves. The growth of bacterial culture was measured in terms of optimal density at 595 nm.

16. Prediction of Bacterial chromium accumulation and deposition site by Transmission Electron Microscope (TEM):

The cells were pelleted down from 10 mM hexavalent chromium supplemented media, were properly washed and fixed using fixative [3% (v/v) glutaraldehyde in 0.1 M PBS] and subsequently washed thrice with the 0.1 M saline phosphate buffer (PBS). The cell pellets were stained with 1% osmium tetraoxide, and 2% UranyLess (Electron Microscopy Sciences) [Zakaria et al. 2007]. After washing and properly removing the formed artifacts with sterile millipore water, sequentially dehydrated samples were applied to the copper grid, dried, and viewed under TEM (JEOL JEM 2100 with EELS).

17. Observe the effect of chromium on bacterial cell wall by Scanning Electron Microscope (SEM):

The cell biomass were isolated by centrifuging the mid log phase cells and cell pellets were properly washed and fixed with 3% (v/v) glutaraldehyde and then washed thrice with the 0.1 M PBS. Following sequential dehydration, the samples were applied on the cover slip, dried, and SEM micrographs were observed after platinum coating using Zeiss Evo-MA version 10.

18. Optimization of bacterial metal absorbance by batch chromium absorption and removal assay:

To conduct the experiment, 20 ml of 10 mM and 40 mM chromium supplemented media were inoculated with 10% bacterial batch culture. After specific time intervals, the microbial cell was collected from the metal supplemented batches and was centrifuged at 5000 rpm for 10 minutes, and residual metal concentration of filtrate and properly washed (with sterile millipore water) cell pellet were monitored using EDXRF spectrometer and AAS.

To estimate the bacterial metal retention efficiency, washed the cell pellets properly with sterile millipore water and reintroduced the washed pellets into metal free LB broth for three more times (for 24 hours) and residual metal content of the cell free culture filtrate and pellet was measured batch to batch after specific time intervals.

19. Study of PGPR (Plant Growth Promoting Rhizospheric bacteria) like traits:

To monitor Indole compound formation, the bacteria were cultured in 1 mg/ml of L-tryptophan supplemented Luria Bertani Broth media. After 24 hours of incubation, 1 ml of bacterial culture was centrifuged, and ferric chloride-perchloric (Salkowski reagent; $FeCl_3$-$HClO_4$) [Gordon and Weber 1951] was added in a 1:1 ratio to the supernatant and incubated for 30 minutes in dark. The colour intensity of the supernatant was measured by a spectrophotometer using the 530 nm wavelength. The amount of Indole compound produced was measured by a standard curve provided by Sigma Aldrich. The concentrations of different indole-containing compounds (IAA and IBA) were confirmed by HPTLC (High-performance thin-layer chromatography) using isopropanol, ammonia, and distilled water in the ratio 8:1.4:0.6 as the mobile phase, with 10 cm/10 cm silica plates of 200 µl layer thickness, and was executed on TLC scanner 3.

Bacterial siderophore production was measured by Chrome Azurol S agar (CAS agar) test, $FeCl_3$ test, and estimation of bacterial total cell protein content.

CAS agar plates were prepared by dissolving Chrome Azurol S and hexadecyltrimethyl ammonium bromide in King's B base. The disappearance of the blue colour of Fe^{3+} and the formation of orange pigment due to the extraction of siderophore by the iron-starved microbes were observed after 2 to 7 days of incubation.

The $FeCl_3$ test was performed by adding 1 ml (to detect hydroxamate siderophore**)** to 3 ml (to detect catechol siderophore) of freshly prepared 2% $FeCl_3$ to 1 ml of cell free culture filtrate, and the intensity of the purplish brown colour was detected by the appearance of 420-450 nm spectrophotometric peaks [Jalal and Vander 1990].

Bacterial total cell protein content was estimated by the conventional Lowry method [Lowry 1951] to calculate the amount of hydroxamate siderophore produced per mg of cellular protein.

To estimate microbial phosphate-solubilizing enzyme producing ability, inspect the appearance of the clear zone surrounding the bacterial colonies that were retained on Pikovskaya agar plates, after five days of incubation.

20. Grown plant in experimental condition:

To inspect Bio-concentration factor of plants grown in the presence of the pure and mixed culture of isolated soil bacteria, and comparing it with plants collected from sewage regions, the *Vigna radiata* seedlings were grown in soil supplemented 10 mM hexavalent chromium. Mixed microbial culture of East Kolkata Wetland, Circular Canal, Kestopur Khal and pure culture of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018, and the *Bacillus xiamenensis* 26K018 were applied at 24 hour intervals to contaminate the experimental soils. A control set was maintained where plants grown in hexavalent chromium supplemented sterile soil.

21. Calculation of plant Bio-absorption factor (BCF):

The BCF of plant rhizospheric soils is the ratio of the metal concentration in plant to the metal concentration in the soil supporting that vegetation. A <1 BCF indicates that the plant likely absorb the heavy metal, but do not accumulate it within the cell.

22. Statistical analysis:

Each experiment was repeated in triplicate. The data were standardized using one way ANOVA and two tailed t test. Significant differences were compared using at $P \leq 0.05$ significance level

(and $p < 0.01$ high significance level) and r value between +1 and -1 using Microsoft Excel and SPSS software.

V. Result:

As the initial characterization was done before the ability of the selected strains to remove chromium was tested, results of objective II are being described first followed by those from objective I.

Objective II: Community composition among bio-remediating microbes present in different wetlands of Kolkata and molecular profiling:

Physico-chemical nature of soil:

Inspecting the character of the soil is important because the basic soil physiology is the relevant indicator of the soil microbial community diversity [Tripathi et al. 2013; Lauber et al. 2009]. The sewage soil of Kestopur Khal and Tolly Nullah was blackish, while that of East Kolkata Wetland was grayish in colour (Figure 2).

[2A] **[2B]** **[2C]**

Figure 2: Soil samples collected from different sewage sites (2A: East Kolkata Wetland; 2B: Circular Canal; 2C: Kestopur Khal)

Different soil physico-chemical parameters like soil pH, Dissolved Oxygen (DO), Chemical Oxygen Demand (COD), Dehydrogenase activity (DHA) was measured and compared (figure 3).

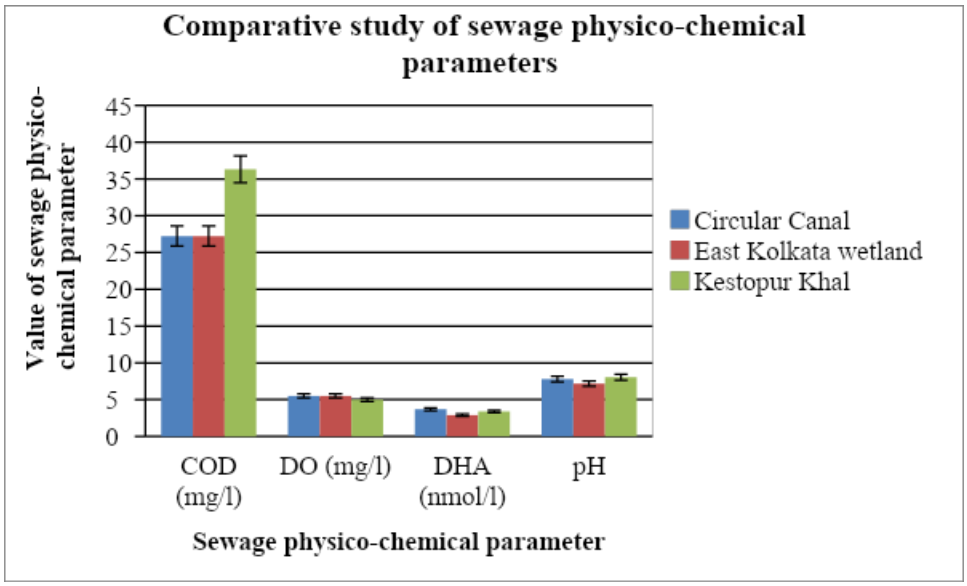


Figure 3: Comparative study of different soil physico-chemical parameters from different sewage sites

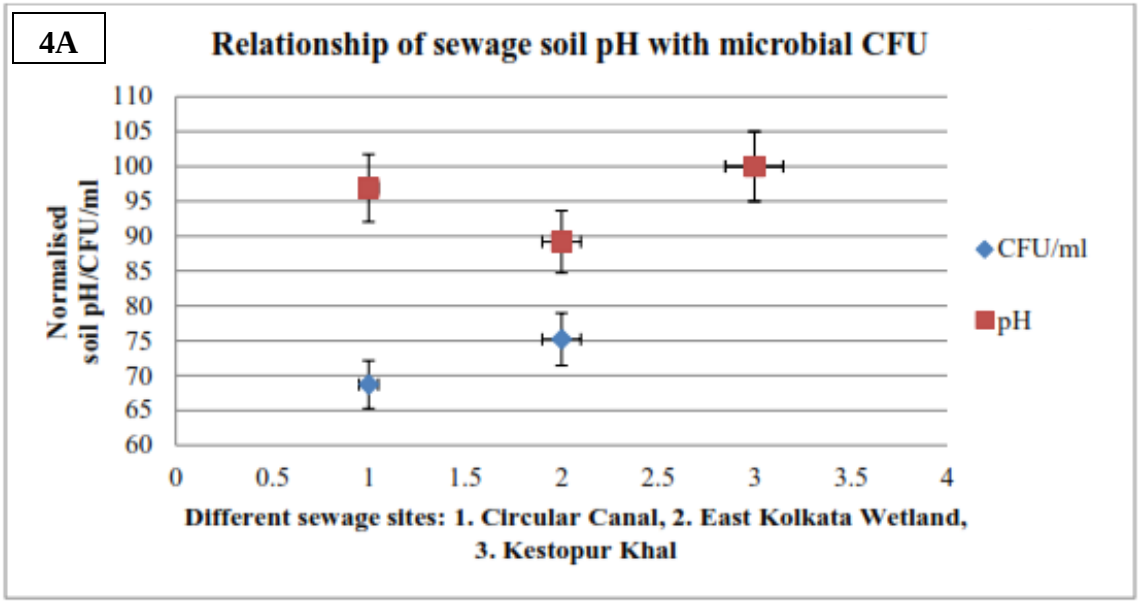
The pH of the soil samples collected from the different sewage regions was found to be neutral to slightly alkaline (7.8±0.05, 7.18±0.25, and 8.05±0.02 at Circular Canal, East Kolkata Wetland, and Kestopur Khal, respectively). Generally, pH values ranging between 4.5 and 8.3 are optimum for microbial growth owing to the suitability of mineral constituents and the availability of carbon source and our result shown that within the optimal pH range number of bacterial colonies was risen gradually (Figure 4A)

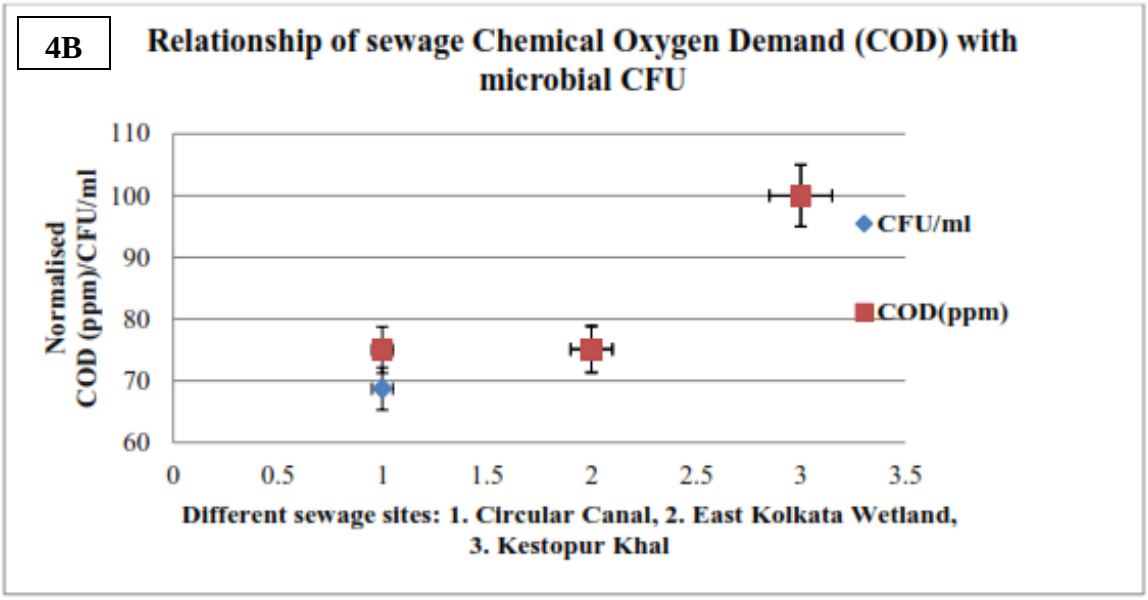
COD of sewage is the amount of oxygen consumed mainly by the sewage bacteria. The COD of the sewage effluent of Kestopur Khal was much greater (36.3±1.1 mg/l) than those of the effluents collected from the two other sewage regions (27.25±0.9, 27.25±1.3 mg/l), indicating that the soil of Kestopur Khal is more polluted than those of the other two regions. The results revealed that the sewage sample of Kestopur Khal contained a greater amount of oxidizable organic material (Figure 4B).

The concentration of DO in the sewage water of Circular Canal, East Kolkata Wetland and Kestopur Khal was 5.5±0.01 mg/l, 5.5±0.5 mg/l and 5±0.001 mg/l, respectively. Amount of DO

reduced gradually with microbial growth because microbes utilized it to decompose organic matter (Figure 4C).

The value of soil DHA was much greater at Circular Canal and Kestopur Khal (3.7±0.9 nmol/l and 3.4±1.0 nmol/l, respectively) than at East Kolkata Wetland (2.9±0.7 nmol/l). Lower soil DHA indicated that the three wastewater bodies herein were contaminated with heavy metals and amongst the three; East Kolkata Wetland was highly polluted (Figure 4D).





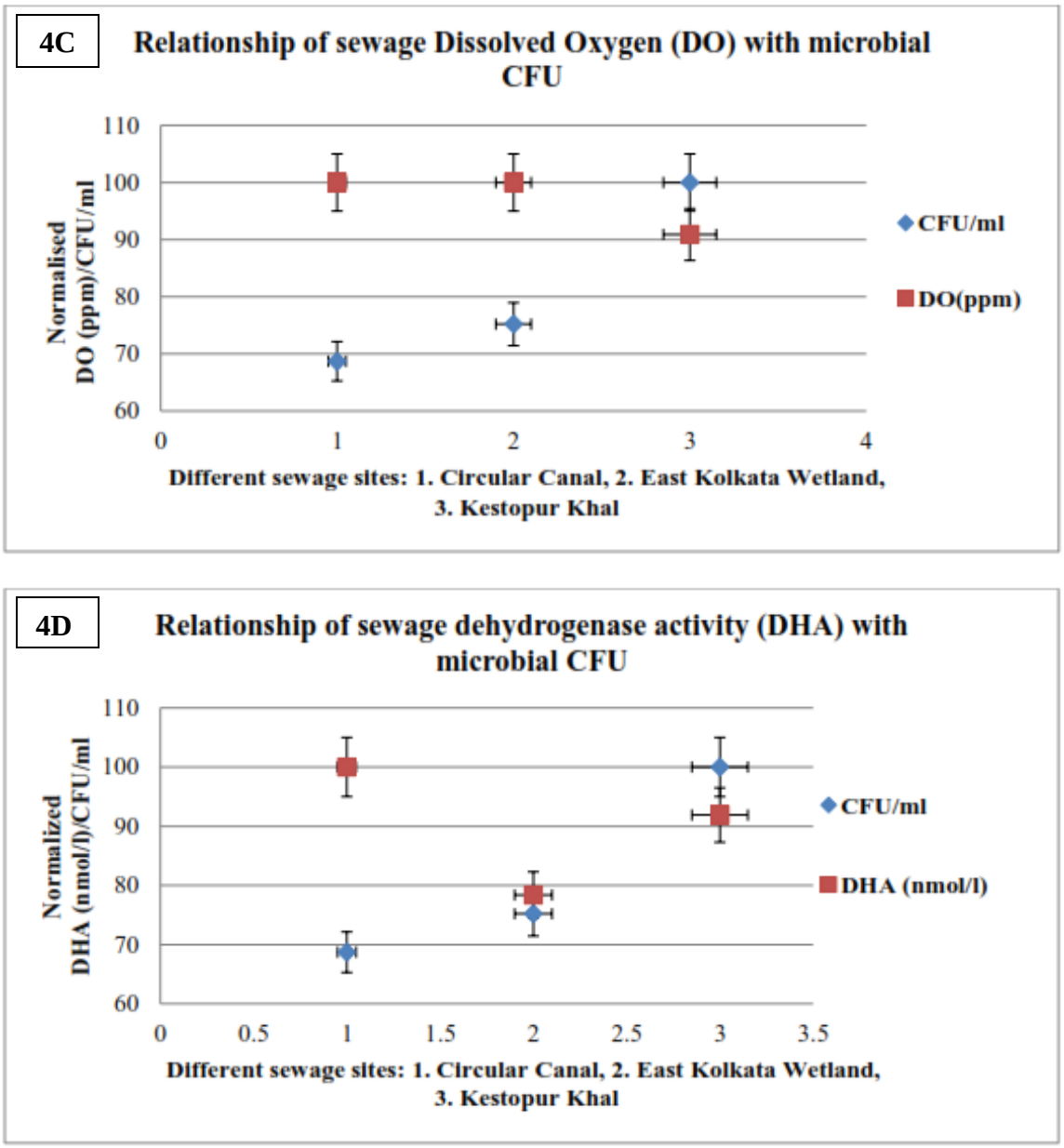


Figure 4(A-D): Relationship of different soil physico-chemical characters (normalized) with bacterial Colony Forming Unit (CFU/ml) shown that some parameters (pH and COD) had related positively [4A & 4B] and some others (DO and DHA) had negatively related [4C & 4D].

The results of Figure 4 indicate that the in soil samples collected from 6-10 cm depth of three different sewage sites of Kolkata, bacterial CFU is positively correlated with pH (r=0.6; p=0.5) and COD (r=0.99; p=0.013) and negatively correlated with DO (r=-0.99; p=0.013) and DHA (r=0.12; p=0.9).

Characterization of soil microbes isolated from different sewage sites:

The microbes isolated from different sewage regions were differentiated in terms of colony characteristics, including colony form, elevation, surface, edge, colour (Figure 1). From the properly distinguished single colonies, microbial strains were selected on the basis of their colony morphology. Most of them exhibited the presence of entire edges, smooth surfaces, flat elevation, circular to elongated form, and rapid growth (Table 1).

The cellular morphology of the isolated microbes was determined that most of the bacterial isolates were Gram positive rods and possessed endospore-forming ability (Table 1).

Table 1: Morphological characterization of isolated soil bacteria:

Strain No.	Colony Character		Cell Character		
	Color	Nature	Gram Nature	Shape	Endospore
1-C	Straw yellow	Rapid, entire, circular, smooth, flat	Gram +Ve	Cocci	Present
2-C	Whish orange	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present
3-C	Orange	Less rapid, entire, circular, rough, flat	Gram +Ve	Rod	Present
4-C	Yellow	Rapid, entire, circular, smooth, flat	Gram –Ve	Cocci	Present
5-C	Whitish yellow	Less rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
6-C	White	Rapid, entire, elongated,	Gram +Ve	Rod	Present

		smooth, flat			
7-C	Whitish yellow	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Rod	Present
8-C	Translucent	Rapid, entire, circular, smooth, concave	Gram –Ve	Rod	Present
9-C	White	Rapid, entire, circular, smooth, flat	Gram +Ve	Cocci	Present
10-C	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Long rod	Present
11C	Yellow	Rapid, entire, circular, smooth, flat	Gram +Ve	Rod	Present
12-C (*Microbacterium radiodurans* K12016)	Grayish white	Rapid, wavy, circular, rough, flat	Gram + Ve	Short Rod	Present
1-E (*Bacillus xiamenensis* strain 1E0018)	Straw Yellow	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present
2-E	White	Less rapid, entire, circular, rough, flat	Gram +Ve	Rod	Present
3-E	White	Rapid, entire, circular, smooth, flat	Gram +Ve	Rod	Present
4-E	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Cocci	Present
5-E	Whitish Yellow	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
6-E	White	Rapid, entire, circular, smooth, flat	Gram -Ve	Cocci	Present
7-E	Whitish	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present

8-E	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
9-E	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
10-E	White	Rapid Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
11-E	White	Rapid, entire, circular, smooth, concave	Gram -Ve	Rod	Present
12-E	White	Rapid, entire, circular, smooth, flat	Gram +Ve	Rod	Present
13-E	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
14-E	White	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Cocci	Present
15-E	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Cocci	Present
1-K	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Cocci	Present
2-K	White	Rapid, entire, circular, smooth, convex	Gram +Ve	Rod	Present
3-K	Translucent	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
4-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
5-K	White	Rapid, entire, circular, smooth, flat	Gram +Ve	Rod	Present
6-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
7-K	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present

8-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Rod	Present
9-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
10-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
11-K	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present
12-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
13-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
14-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Rod	Present
15-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Cocci	Present
16-K	White	Rapid, entire, circular, smooth, concave	Gram +Ve	Rod	Present
17-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
18-K	White	Rapid, entire, elongated, smooth, flat	Gram +Ve	Rod	Present
25-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Rod	Present
26-K (*Bacillus xiamenensis* strain 26K018)	Greyish White	Rapid, slightly wavy, circular, smooth, flat	Gram +Ve	Rod	Present

The results of biochemical characterization revealed that the majority of the isolated microbial strains produced catalase and α-amylase enzymes, along with the carotenoid pigment of extracellular origin, and had participated in the mixed acid fermentation test (Table 2).

Table 2 (A-C): Biochemical characters of the isolated soil bacteria ("+" denotes the enzyme producing property of the isolate and "–" denotes the absence of the enzyme producing property):

A. Bacteria Isolated from Circular Canal (**Microbacterium radiodurans* **K12016)**

Biochemical Tests	**Bacterial strains**											
	1-C	**2-C**	**3-C**	**4-C**	**5-C**	**6-C**	**7-C**	**8-C**	**-9C**	**10-C**	**11-C**	**12-C***
1.Gelatinase Test	-	+	+	-	-	-	+	-	-	-	+	-
2. Catalase Test	+	+	-	+	+	+	+	+	-	+	+	+
3. Amylase Test	+	+	-	+	+	++	+	+	+	+	+++	+
4. Fluorescence Test	-	-	-	-	-	-	-	-	-	-	-	-
5. Methyl red Test	++	+++	+	+	+	+	+++	+	+++	+	+	+
7. Urease Test	+	+	-	+	+	-	+	-	-	-	-	-
8. Diffusible pigmentation Test												
After 1 days	-	+	-	-	+	+	-	-	-	-	-	-
After 6 days	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	+++

B. Bacteria isolated from East Kolkata Wetland (**Bacillus xiamenensis* **strain 1E0018)**

Biochemical Tests	**Bacterial strains**											
	1-E*	**2-E**	**3-E**	**4-E**	**5-E**	**6-E**	**7-E**	**8-E**	**9-E**	**10-E**	**11-E**	**12 -E**
1.Gelatinase Test	-	-	-	-	-	-	-	+	-	-	-	-
2. Catalase Test	-	-	-	-	+	-	-	-	-	-	-	-
3. Amylase Test	+	+	+	+	+	+	+	+	-	+	+	+
4. Fluorescence Test	-	-	-	-	-	-	-	-	-	-	-	-
5. Methyl red Test	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++
7. Urease Test	-	-	-	-	-	-	-	+	-	-	-	-
8. Diffusible pigmentation Test												
After 1 days	-	-	-	-	-	-	-	-	-	-	-	-
After 6 days	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	+++

C. Bacteria isolated from Kestopur Canal (*_Bacillus xiamenensis_ strain 26K018)

Biochemical Tests	Bacterial strains											
	1-K	2-K	3-K	4-K	5-K	6-K	7-K	8-K	11-K	12-K	25-K	26-K*
1.Gelatinase Test	+	++	+	+	+	+	-	-	-	-	-	-
2. Catalase Test	-	-	-	-	+	+	-	-	-	-	-	-
3. Amylase Test	+++	+++	+++	+++	+++	-	+++	+++	+++	+++	+	+++
4. Fluorescence Test	-	-	-	-	-	-	+	-	-	-	+	-
5. Methyl red Test	+++	-	-	+++	-	-	+++	-	-	-	-	++
7. Urease Test	-	-	-	-	-	+++	-	-	-	+	-	-
8. Diffusible pigmentation Test												
After 1 days	-	-	-	-	-	-	-	-	-	-	-	+
After 6 days	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

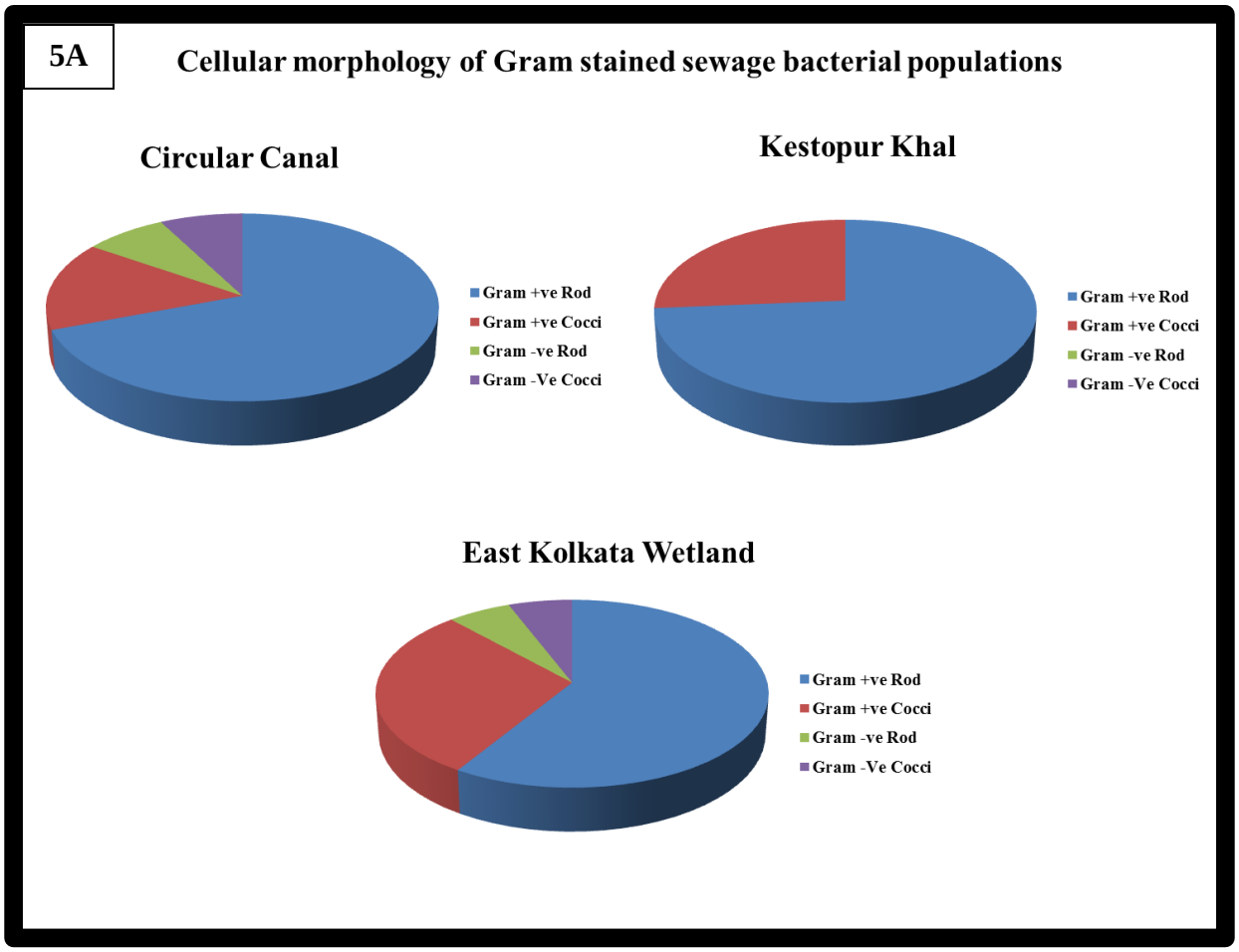
The antibiotic profiling data indicated that most of the isolated soil microbes were sensitive to ampicillin (Table 3).

Table 3: Microbial antibiotic sensitivity (µg/ml) index in Muller Hinton Agar [The antibiotic concentration (in µg) was measured before applying it to disc]:

Bacterial Strain	Antibiotics			
	Ampicillin (10 µg/ml)	**Tetracycline (30 µg/ml)**	**Deoxycyclin (30 µg/ml)**	**Norfloxacin (10 µg/ml)**
1-C	Sensitive	Resistant	Intermediate	Resistant
2-C	Intermediate	Resistant	Resistant	Resistant
3-C	Intermediate	Resistant	Sensitive	Intermediate
4-C	Sensitive	Intermediate	Resistant	Resistant
5-C	Sensitive	Resistant	Intermediate	Resistant
6-C	Intermediate	Intermediate	Sensitive	Intermediate
7-C	Sensitive	Intermediate	Intermediate	Resistant
8-C	Intermediate	Sensitive	Intermediate	Sensitive
9-C	Sensitive	Intermediate	Sensitive	Intermediate
10-C	Sensitive	Resistant	Resistant	Resistant
11-C	Resistant	Resistant	Resistant	Intermediate
12-C (*Microbacterium radiodurans* K12016)	Sensitive	Resistant	Intermediate	Resistant
1E(*Bacillus xiamenensis* strain 1E0018)	Sensitive	Intermediate	Resistant	Intermediate
2-E	Intermediate	Sensitive	Intermediate	Resistant
3-E	Sensitive	Intermediate	Intermediate	Resistant
4-E	Intermediate	Resistant	Intermediate	Resistant
5-E	Resistant	Resistant	Resistant	Sensitive
6-E	Sensitive	Intermediate	Intermediate	Sensitive
7-E	Sensitive	Resistant	Resistant	Resistant
8-E	Sensitive	Resistant	Intermediate	Resistant
9-E	Intermediate	Resistant	Resistant	Resistant
10-E	Sensitive	Resistant	Intermediate	Sensitive
11-E	Resistant	Intermediate	Intermediate	Sensitive
12-E	Sensitive	Intermediate	Resistant	Resistant
13-E	Sensitive	Intermediate	Sensitive	Sensitive
14-E	Resistant	Resistant	Sensitive	Sensitive

15-E	Sensitive	Resistant	Sensitive	Resistant
1-K	Sensitive	Resistant	Intermediate	Sensitive
2-K	Sensitive	Resistant	Sensitive	Sensitive
3-K	Sensitive	Resistant	Intermediate	Resistant
4-K	Sensitive	Resistant	Sensitive	Intermediate
5-K	Intermediate	Intermediate	Intermediate	Sensitive
6-K	Sensitive	Resistant	Resistant	Resistant
7-K	Sensitive	Resistant	Resistant	Intermediate
8-K	Sensitive	Resistant	Resistant	Sensitive
9-K	Intermediate	Intermediate	Sensitive	Sensitive
10-K	Sensitive	Resistant	Sensitive	Sensitive
11-K	Sensitive	Intermediate	Sensitive	Intermediate
12-K	Sensitive	Intermediate	Intermediate	Resistant
13-K	Sensitive	Intermediate	Resistant	Intermediate
14-K	Sensitive	Resistant	Resistant	Resistant
15-K	Intermediate	Resistant	Sensitive	Sensitive
16-K	Sensitive	Resistant	Resistant	Resistant
17-K	Sensitive	Intermediate	Sensitive	Resistant
18-K	Intermediate	Resistant	Sensitive	Resistant
25-K	Intermediate	Resistant	Resistant	Intermediate
26-K (*Bacillus xiamenensis* strain 26K018)	Intermediate	Intermediate	Sensitive	Intermediate

The microbial populations of three different sewage regions were more or less similar in morphology (Figure 5A) and showed the same trend in physiological and biochemical parameters (Figure 5B).


5A
Cellular morphology of Gram stained sewage bacterial populations
Circular Canal
Gram +ve Rod
Gram +ve Cocci
Gram -ve Rod
Gram -Ve Cocci
Kestopur Khal
Gram +ve Rod
Gram +ve Cocci
Gram -ve Rod
Gram -Ve Cocci
East Kolkata Wetland
Gram +ve Rod
Gram +ve Cocci
Gram -ve Rod
Gram -Ve Cocci

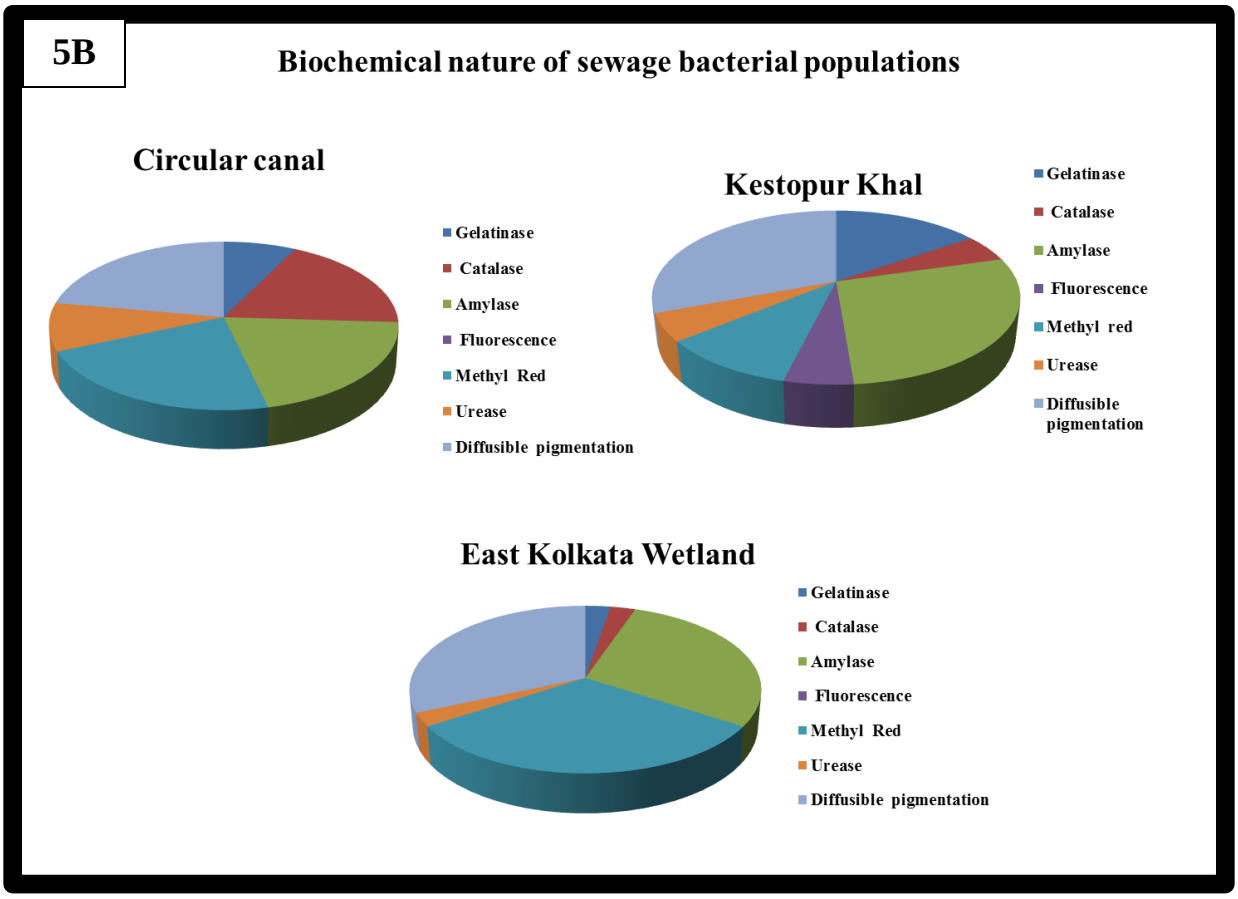


Figure 5: Comparative evaluation of [A] morphological and [B] biochemical characters of microbes isolated from sewage regions of Kolkata

Optimum growth condition for most of the microbes was 37°C temperature (figure 6A) and neutral pH (pH 7) (Figure 6B).

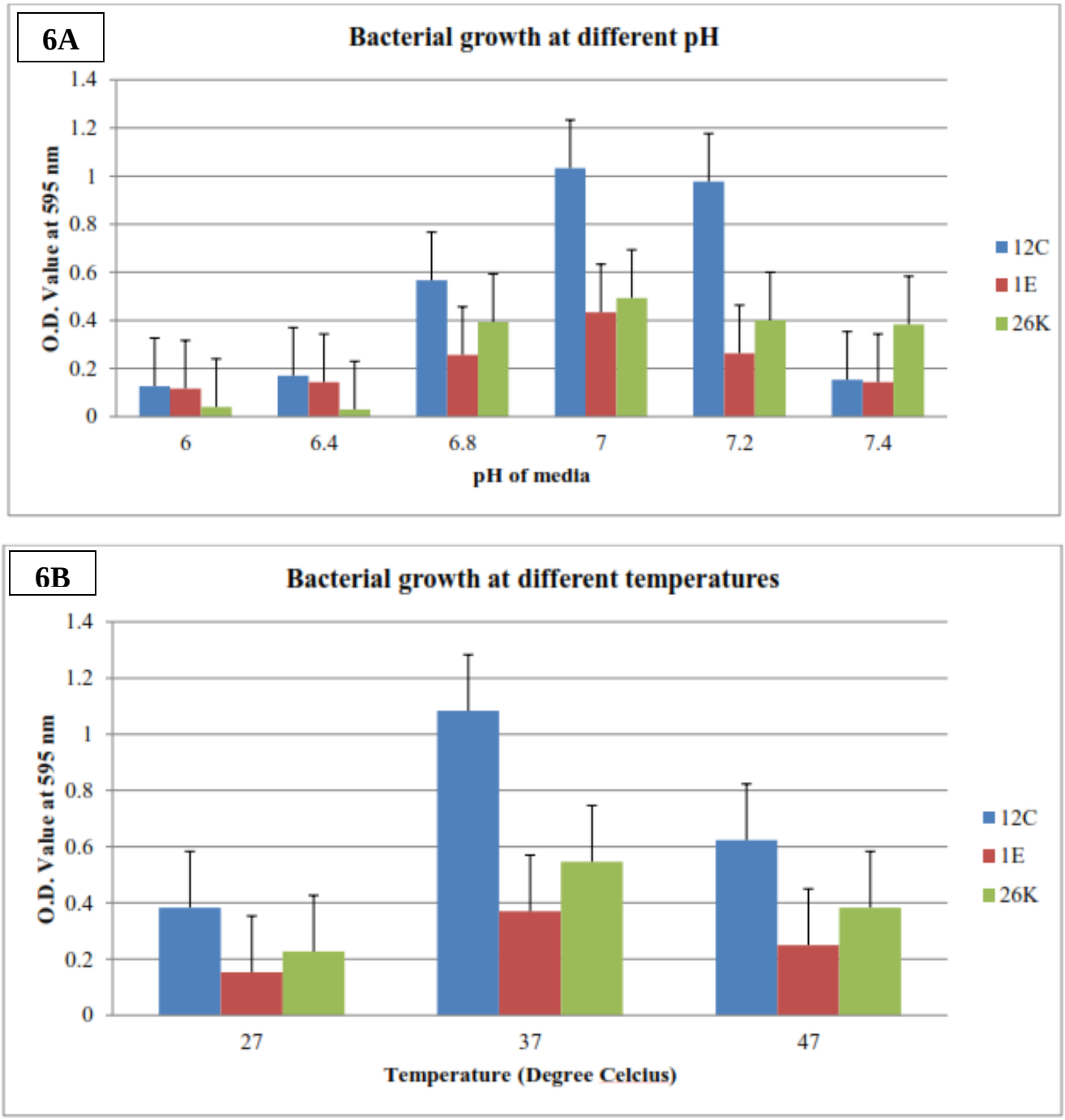


Figure 6 (A-B): Standardization the growth conditions of some selected microbes by optimizing the different important growth parameters like pH, temperature
(12C: *Microbacterium radiodurans* K12016; 1E: *Bacillus xiamenensis* strain 1E0018; 26K: *Bacillus xiamenensis* strain 1E0018)

Molecular characterization of microbe:

Three microbial strains (one from each site) shown Cr^{6+} resistance afterwards, were thoroughly studied to find their chromium removing capacity and identified by 16S rDNA sequencing.

The 16S rDNA of the strain 12C (isolated from the Circular Canal), 1E (isolated from East Kolkata Wetland), and 26K (isolated from Kestopur Khal), were sequenced, identified by sequence alignment and phylogenetic analyses with their near relatives (Figure 7, Figure 8) and their taxonomic positions were further identified by standard identification hierarchy. The 12C strain had 91.01% sequence identity with *Microbacterium radiodurans* N28_137 (Table 4). Zhang *et al.* (2010) previously reported one UV radiation resistant strain of *Microbacterium radiodurans* from soil. There are different chromium and other heavy metal removing *Microbacterium* species but according to our literature review no report found earlier regarding chromium resistance property of *Microbacterium radiodurans*. Since our finding indicates that *Microbacterium radiodurans* K12016 absorbs chromium from surroundings, it may play an important role in chromium bioremediation. Both the 1E and 26K strains had 99% similarity with the MCCC 1A00008 strain of *Bacillus xiamenensis* (Table 5 & Table 6). Preliminary morphological, biochemical, physiological characterization and taxonomic key analysis also confirmed these reports. The newly annotated partial 16S rRNA sequences of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 was submitted to the NCBI database with the GenBank accession number MF600628, MK353157 and MK353500. Previous study by Gupta et al (2012) was also reported that most of the metal resistant soil microbes isolated from Kestopur Khal were different species of *Bacillus* and some are *Exiguobacterium* (accession numbers JN392001-JN3920013). Our present study confirmed the presence of different species of *Bacillus* as the most predominant bacteria in the microbial community of different wastelands of Kolkata (Table 7, Figure 9). Most of the microbial populations from three previously mentioned sewage regions of Kolkata possess gram positive rod shaped bacteria.

Table 4: The sequences selected from NCBI genbank database based on maximum identity score with the *Microbacterium radiodurans* K12016 and aligned individually using NCBI MEGABLAST algorithm to find sequence similarity index:

Sequence	% Identity
Microbacterium radiodurans N28 137	91.01
Microbacterium radiodurans N28 139	88.71
Desulfovibrio vulgaris	74.05
Desulfovibrio vulgaris Hildenborough	73.04
Bacillus acidicola 105-2	72.9
Ochrobactrum tritici	78.52
Bacillus cereus 6+	72.33
Microbacterium arborescens S6	73.95
Microbacterium arborescens S3-91	86.52
Microbacterium arborescens S1-81	90.15
Microbacterium arborescens MFB-39 16S	90.34
Cellulosimicrobium cellulans IARI-BHI-13	90.13
Microbacterium paraoxydans 6U	90.55
Microbacterium sp. DP6-560-600	90.34
Microbacterium arborescens MS4	90.55
Microbacterium flavum E2 AS-19	90.13

Marine_bacterium AK6 009	90.34
Marine_bacterium AK6 007	90.34
Marine_bacterium AK6 006	90.34
Microbacterium oleivorans BAS69	90.72

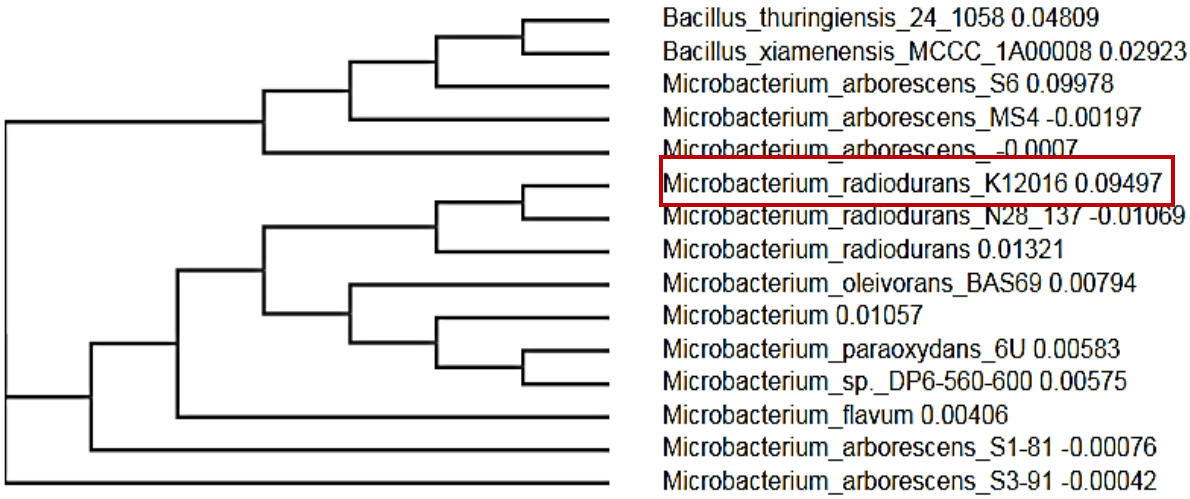


Figure 7: To identify the microbe of interest isolated from sewage soil of Circular Canal, phylogenetic tree was generated using the Clustal Omega multiple sequence alignment algorithm by applying neighbor joining method based on 16S rDNA sequences. The analysis involved 15 different nucleotide sequences which showed maximum identity, higher query coverage and lower distance matrix (as per Kimura-2-Parameter model) with the newly obtained 16S rRNA sequence.

Table 5: The sequences selected from the NCBI genbank database based on maximum identity score with the *Bacillus xiamenensis* 1E0018 and aligned individually using NCBI MEGABLAST algorithms to find sequence similarity index:

Sequence	% Identity
Bacillus xiamenensis MCCC 1A00008	99.85
Bacillus xiamenensis 26K018	99.7
Bacillus cereus ATCC 14579	94.56
Bacillus mycoides DSM	94.45
Bacillus thuringiensis 24_1058	93.38
Exiguobacterium sp. 6b(2011)	91.31
Bacillus firmus KUCr1	95.67
Lysinibacillus sphaericus DSM	94.02
Bacillus oceanisediminis LNHL3	96

Table 6: The sequences selected from the NCBI genbank database based on maximum identity score with the *Bacillus xiamenensis* 26K018 and aligned individually using NCBI MEGABLAST algorithms to find sequence similarity index:

Sequence	% Identity
Bacillus xiamenensis MCCC 1A00008	99.48
Bacillus xiamenensis 1E0018	99.7
Bacillus cereus ATCC 14579	94.34
Bacillus mycoides DSM	94.16

Bacillus thuringiensis 24_1058	93.17
Exiguobacterium sp. 6b(2011)	89.82
Bacillus firmus KUCr1	95.58
Lysinibacillus sphaericus DSM	93.72
Bacillus oceanisediminis LNHL3	95.76

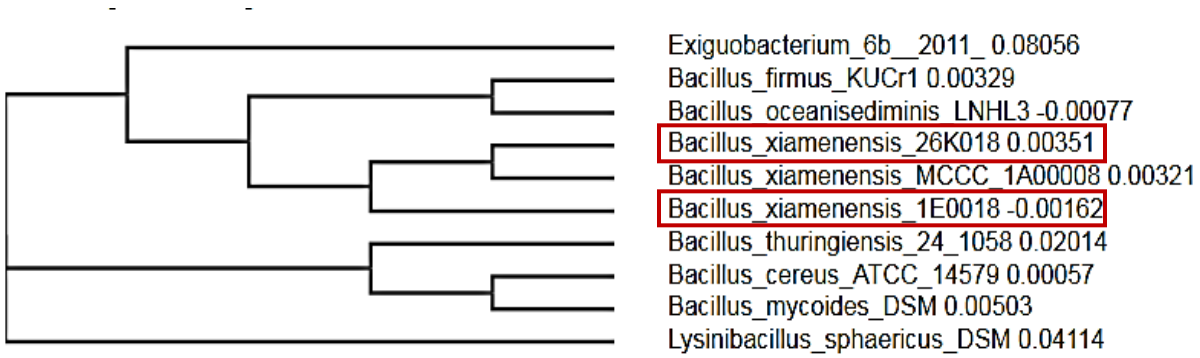


Figure 8: To identify the microbes isolated from sewage soil of East Kolkata Wetland and Kestopur Khal, phylogenetic tree was generated using Clustal Omega multiple sequence alignment algorithm by applying neighbor joining method based on 16S rDNA sequences. The analysis involved 10 different nucleotide sequences which shown maximum identity, higher query coverage and lower distance matrix (as per Kimura-2-Parameter model) with the newly obtained 16S rRNA sequence.

Table 7: The sequences of the isolated soil bacteria were subjected to 16S nucleotide BLAST analysis to find their closest neighbor; the microbial identity was decoded on the basis of the maximum percentage of similarity with their closest neighbors enlisted in the NCBI database.

Strain	Bacterial strain showing maximum homology	Identity (%)	Length (bp)	GenBank Accession number	Collected from
K12016	*Microbacterium radiodurans* strain GIMN 1.002	91.01	708	MF600628	Citcular Canal
1E0018	*Bacillus xiamenensis* strain MCCC IA00008	99.85	1403	MK353500	East Kolkata Wetland
26K018	*Bacillus xiamenensis* strain MCCC IA00008	99.48	1418	MK353157	Kestopur Khal

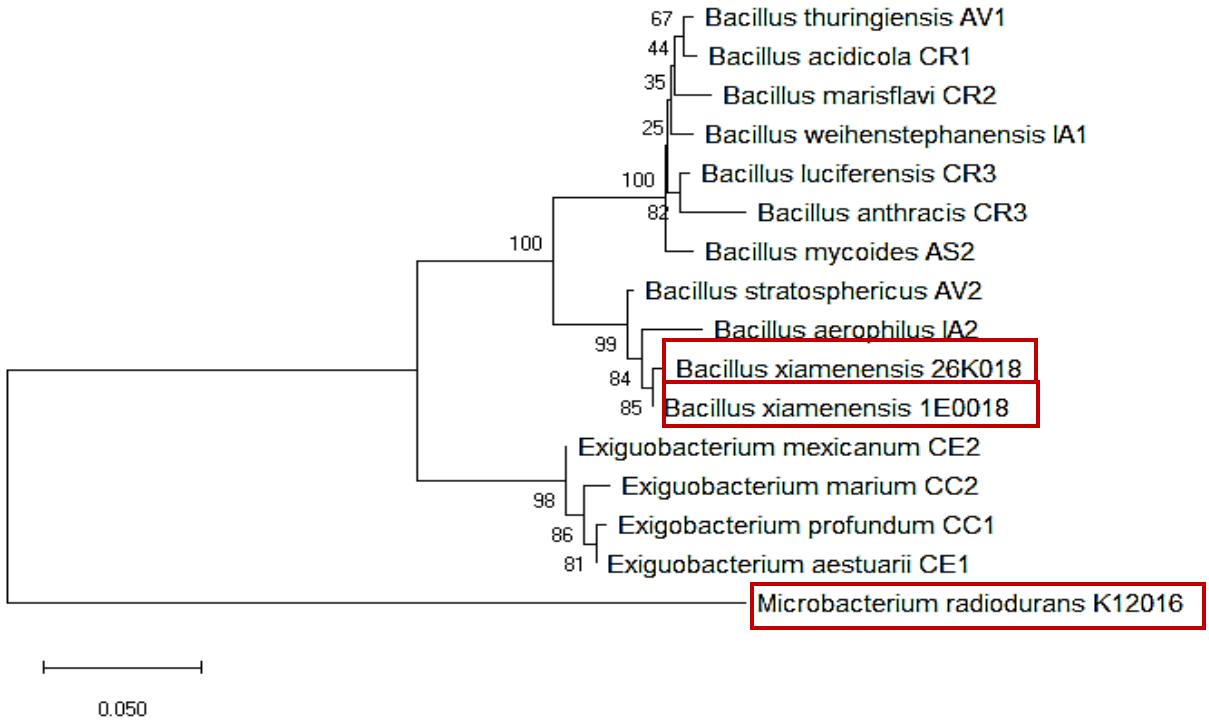


Figure 9. Phylogenetic tree constructed with the strains isolated from Circular canal, East Kolkata Wetland and Kestopur Khal in this study and related strains reported in literature using MEGA 10 software by applying the neighbor-joining method based on 16S rDNA sequences (using 600 bootstrap replicates) implying Kimura 2-parameter method (optimum branch length 0.64059896).

Objective I: Microbial bio-removal efficiencies against heavy metals and their mode of action:

Microbial heavy metal resistance:

Different heavy metals such as nickel, lead, zinc, and chromium were detected in sewage soil by Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer. The chromium content was alarmingly high in the soil of East Kolkata Wetland, being 20864 ± 0.28 ppm, whereas that of the soils collected from Circular Canal and Kestopur Khal were also much greater than normal limit (14-70 ppm according to Health assessment document for chromium, 1984; WHO Air Quality Guidelines – 2nd Ed., 2000), being 187.1 ± 0.41 and 187.1 ± 0.15 ppm, respectively. The concentration of soil chromium also varied greatly among the different sewage regions (Table 8). Chromium was chosen over other metals because we want to observe if any considerable change have taken place in bacterial community composition or bacterial heavy metal removal efficiency because of its huge concentration variability among different sewage regions.

Table 8. Result of soil heavy metal profiling by Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer:

	Chromium (ppm)	**Lead (ppm)**	**Zinc (ppm)**	**Nickel (ppm)**
Circular Canal	**187.1 ± 0.41**	**31.22±0.84**	**238.91±0.67**	**61.22±0.89**
East Kolkata Wetland	**20864 ± 0.28**	**111.73±0.21**	**546.83±0.79**	**56.19±0.67**
Kestopur Khal	**187.1 ± 0.15**	**41.23±0.9**	**381±0.01**	**62.69±0.91**

The results of agar cup technique show that majority of the soil microbes isolated from the bank of three wastelands of Kolkata are resistant up to 10 mM of cadmium and cobalt, but most of them are total resistant (i.e. no zone of inhibition) up to 50 mM hexavalent chromium and 100

mM trivalent chromium respectively (Figure 2). Fifteen (five from each sewage area) isolated microbes namely 5C, 6C, 7C, 8C, 12C, 1E, 2E, 3E, 4E, 7E, 5K, 6K, 7K, 25K and 26K were selected on the basis of their high chromium resistance property from the total fifty four isolates (Table 9).

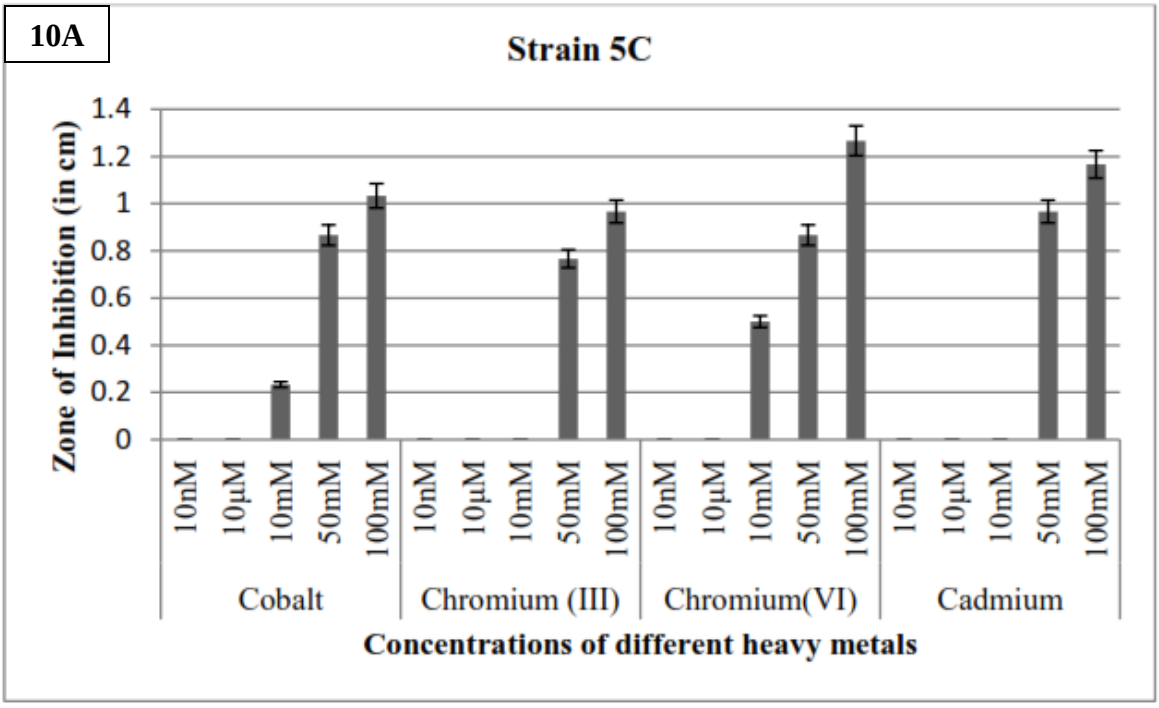
Table 9: Bacterial heavy metal sensitivity (measured by calculating the zone of inhibition)

Strain	Diameter of zone of inhibition against applied heavy metal (in cm)																			
	Cr(III)					Cr(VI)					Cd					Co				
	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM
5C	0	0	0	0.8± 0.05	1± 0.05	0	0	0.5± 0.05	0.9± 0.05	1.3± 0.05	0	0	0	1± 0.05	1.2± 0.05	0	0	0.2± 0.05	0.9± 0.05	1± 0.05
6C	0	0	0	0.7± 0.05	1.1± 0.06	0	0	0	1.5± 0.06	1.8± 0.06	0	0	0.1± 0.05	0.1± 0.05	0.3± 0.05	0	0	0.05± 0.013	0.4± 0.06	0.7± 0.05
7C	0	0	0	0	0.6± 0.06	0	0	0.6± 0.05	1.2± 0.06	1.5± 0.02	0	0	1.3± 0.05	1.3± 0.05	1.5± 0.06	0	0	0.3± 0.06	0.6± 0.05	1.3± 0.06
8C	0	0	0	0	0.6± 0.06	0	0	0	0.6± 0.05	1.1± 0.05	0	0	0	0.8± 0.06	1.3± 0.05	0	0	0.15± 0.09	0.3± 0.05	1± 0.05
12C	0	0	0	0	0	0	0	0	0.3± 0.06	0.7± 0.06	0	0	0	0.7± 0.06	1.1± 0.06	0	0	0	0.6± 0.07	0.7± 0.06
1E	0	0	0	0	0	0	0	0	0.02± 0.002	0.53± 0.05	0	0	1.06± 0.05	1.33± 0.5	1.8± 0.1	0	0	0	0.53± 0.05	0.76± 0.05
2E	0	0	0	0	0	0	0	0	1.26± 0.06	1.6± 0.06	0	0	1.06± 0.06	1.46± 0.06	1.6± 0.06	0	0	0	0.36± 0.06	0.86± 0.06
3E	0	0	0	0	0	0	0	0	0.83± 0.06	1.46± 0.06	0	0	1.06± 0.06	1.43± 0.06	1.66± 0.06	0	0	0	0.53± 0.06	0.86± 0.06
4E	0	0	0	0	0	0	0	0	0.18± 0.05	0.29± 0.05	0	0	0.96± 0.05	1.13± 0.05	1.5± 0.05	0	0	0	0.53± 0.05	0.82± 0.05
7E	0	0	0	0	0	0	0	0	0.21± 0.06	0.55± 0.06	0	0	0	1.06± 0.06	1.18± 0.06	0	0	1.06	1.42± 0.06	1.62± 0.06
5K	0	0	0	0	0	0	0	0	0.56± 0.06	1.3± 0.06	0	0	0.66± 0.06	0.96± 0.06	1.13± 0.06	0	0	0	0.26± 0.06	0.46± 0.06
6K	0	0	0	0	0	0	0	0	0.56± 0.05	1.3± 0.05	0	0.36± 0.05	0.4± 0.05	0.53± 0.05	1.16± 0.05	0	0	0	0	0.16± 0.05
7K	0	0	0	0	0	0	0	0	0.1± 0.06	0.7± 0.06	0	0	0	0	0	0	0	0	0.26± 0.05	0.46± 0.06
25K	0	0	0	0	0	0	0	0	0.13± 0.05	0.16± 0.05	0	0.03± 0.02	0.1± 0.05	0.3± 0.05	0.8± 0.05	0	0	0	0.33± 0.05	0.86± 0.05
26K	0	0	0	0	0	0	0	0.43± 0.05	1± 0.05	2.5± 0.05	0	0	1.06± 0.05	1.26± 0.05	1.53± 0.05	0	0	0	0	0

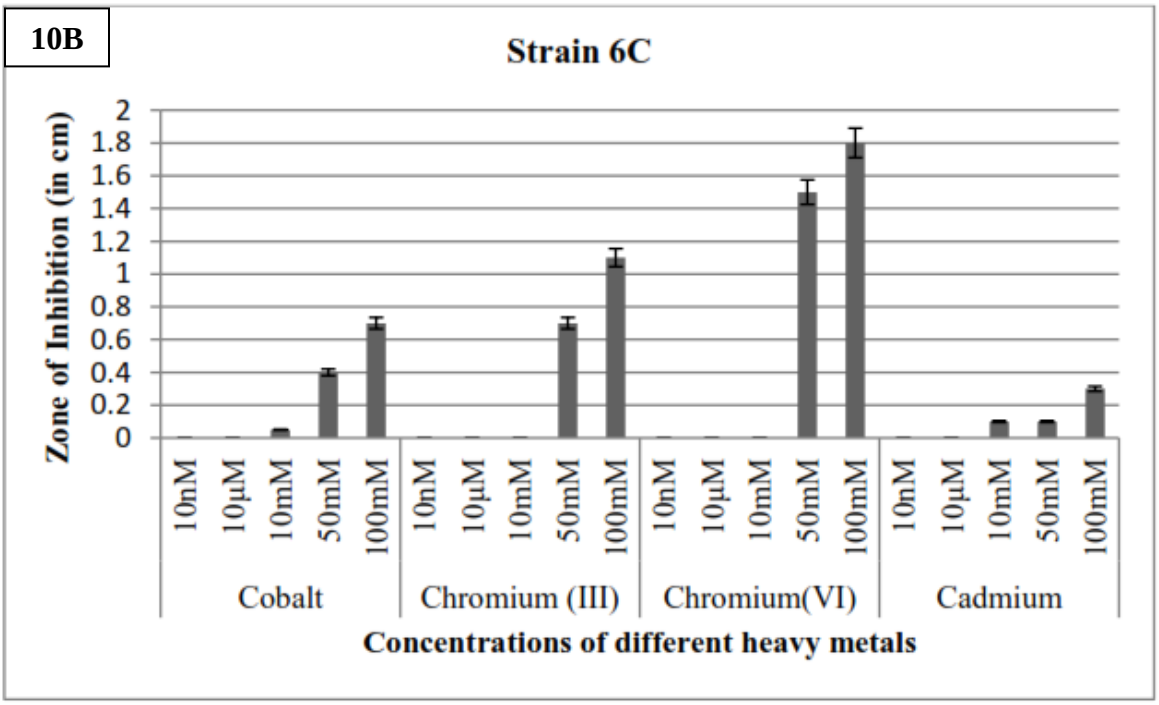
The zone of inhibition measurement for the microbes isolated from Kestopur khal and East Kolkata Wetland show resistance against 100 mM trivalent chromium and 10 mM (26K) and 50 mM hexavalent chromium. The isolates of Circular Canal show resistance for both trivalent and hexavalent chromium of 10 mM to 50 mM concentrations (Figure 10).

I. Bacteria Isolated from Circular Canal

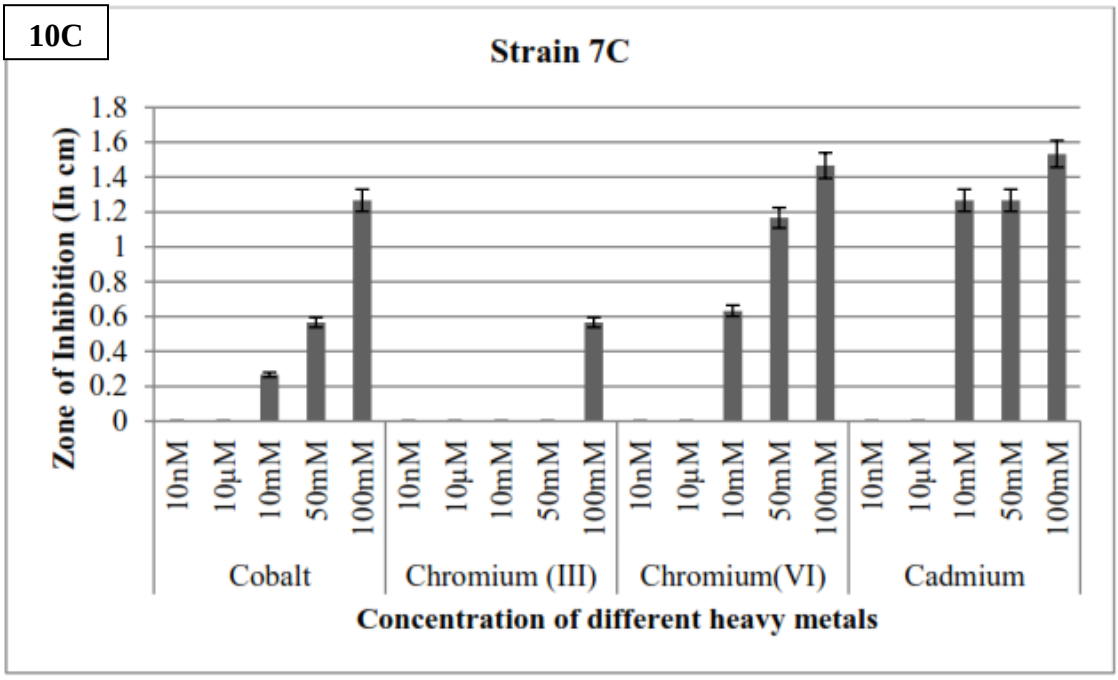
10A



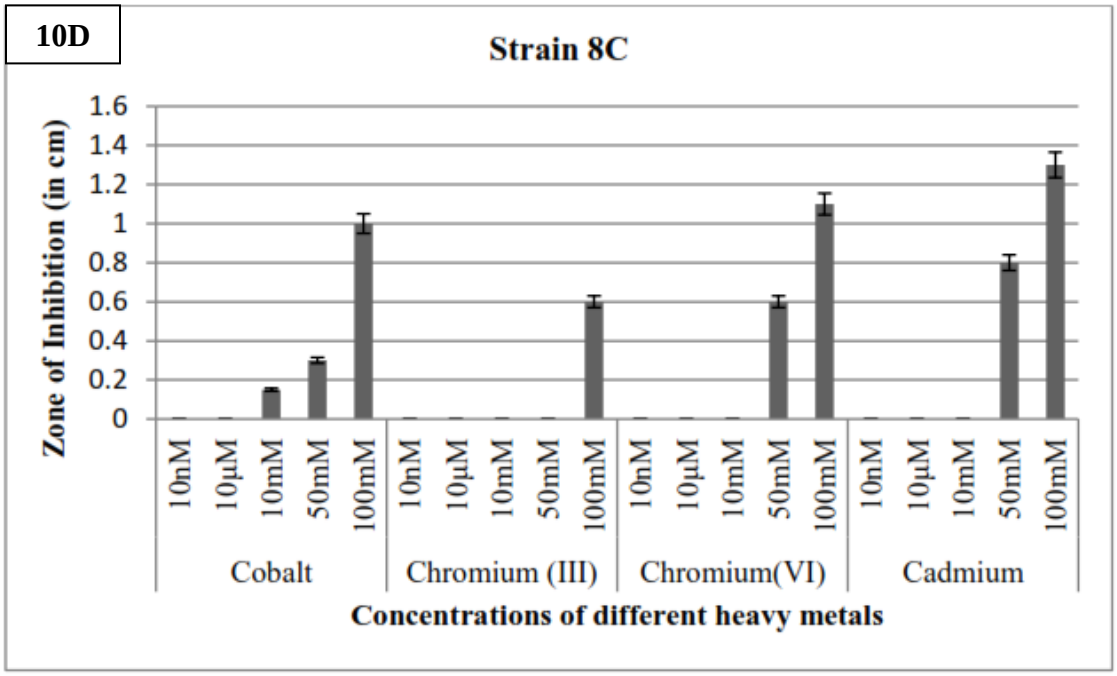
10B

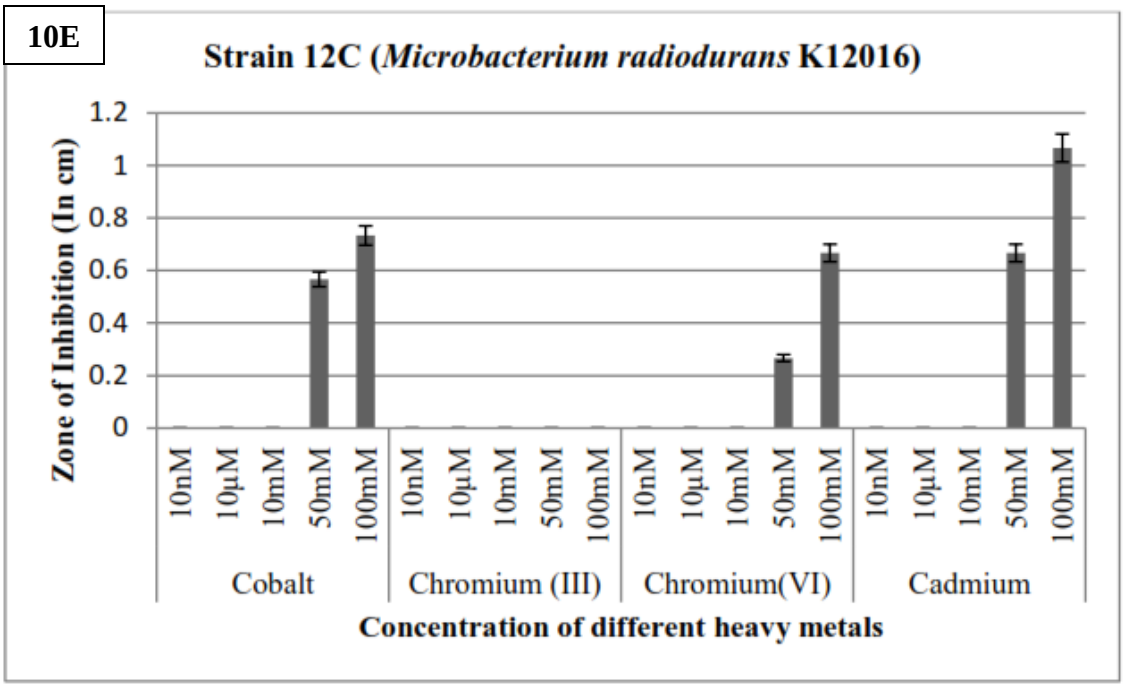


10C

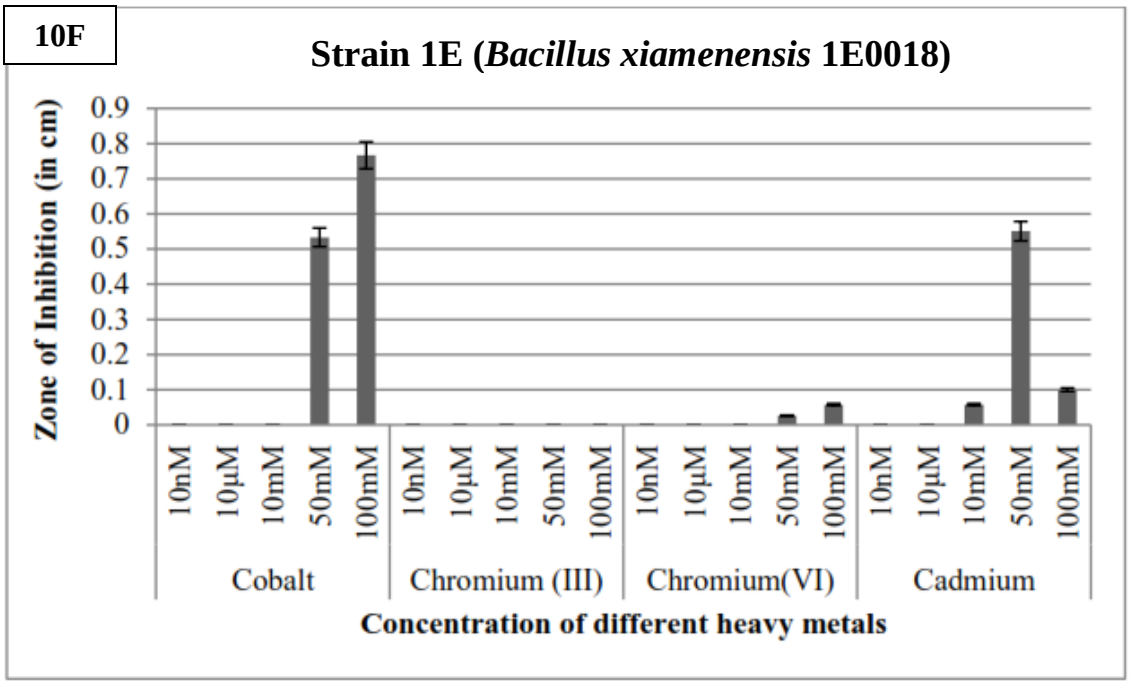

Strain 7C
Zone of Inhibition (In cm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals

10D

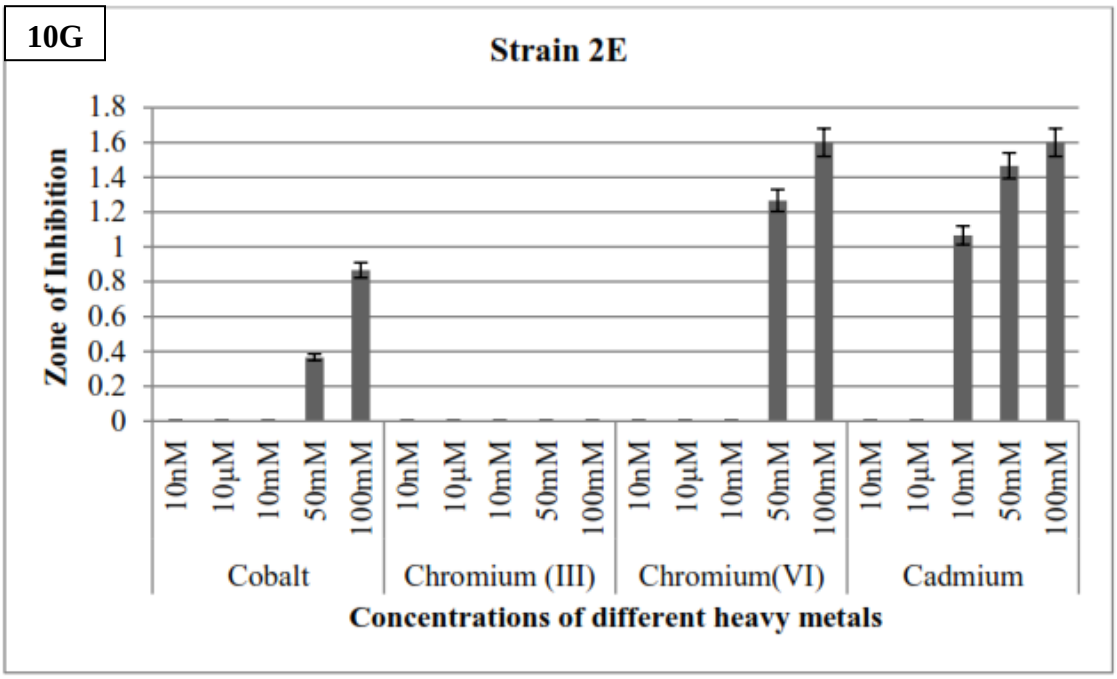

Strain 8C
Zone of Inhibition (in cm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals



II. Bacteria isolated from East Kolkata Wetland



10G


Strain 2E
Zone of Inhibition
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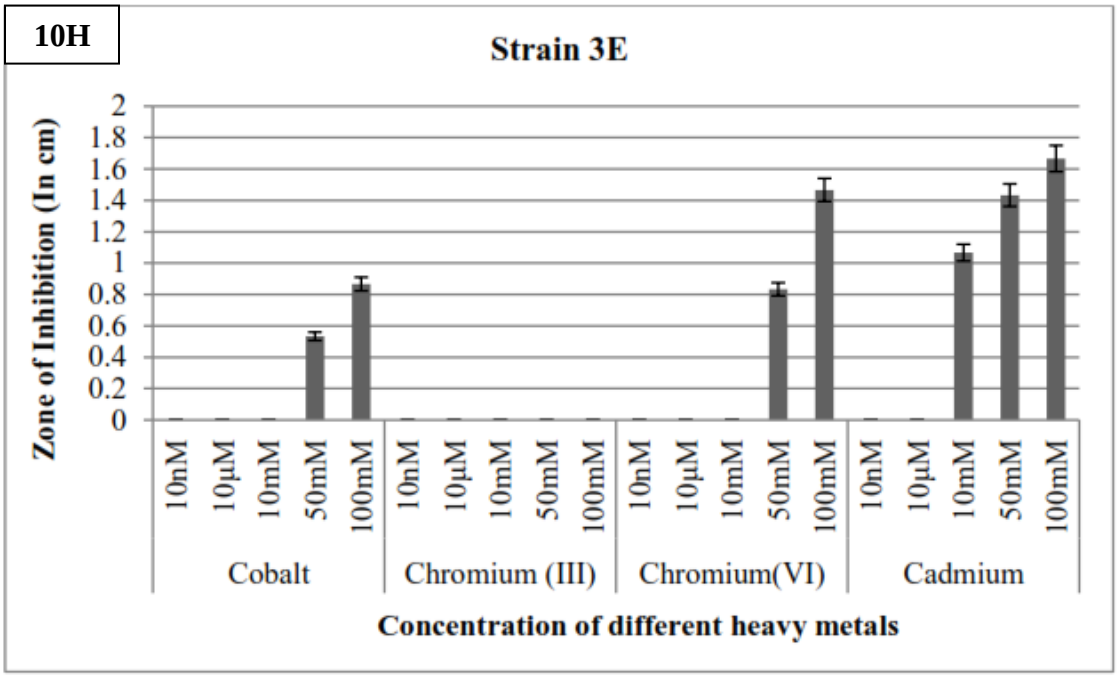
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM
10µM
10mM
50mM
100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals

10H


Strain 3E
Zone of Inhibition (In cm)
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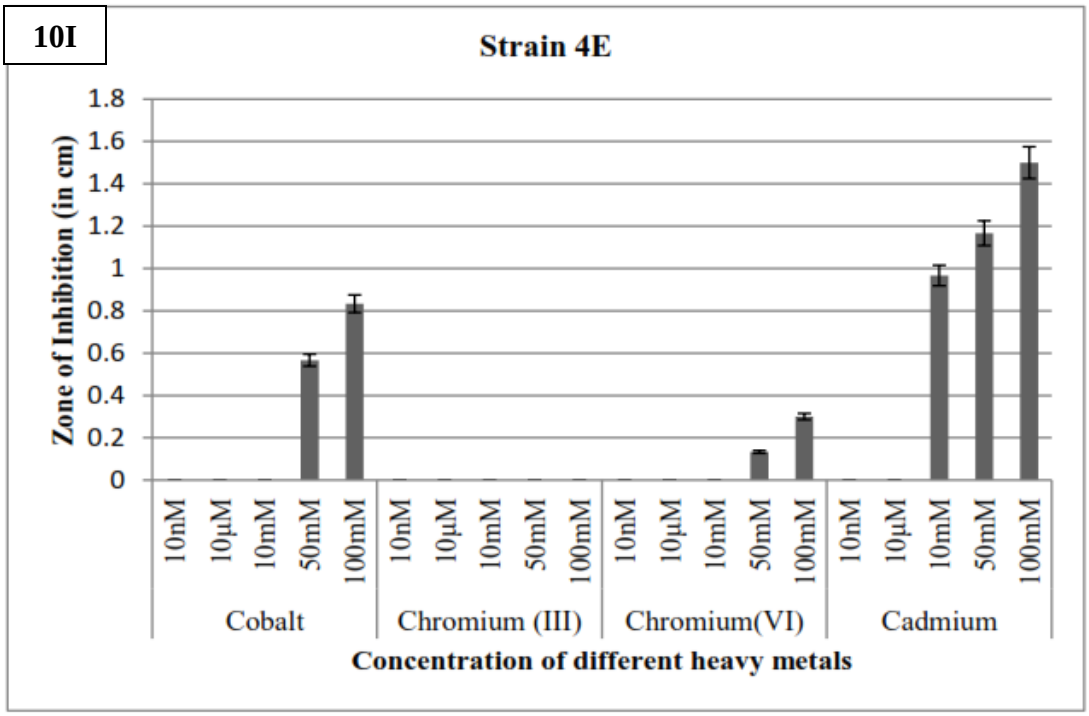
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM
10µM
10mM
50mM
100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals

10I


Strain 4E
Zone of Inhibition (in cm)
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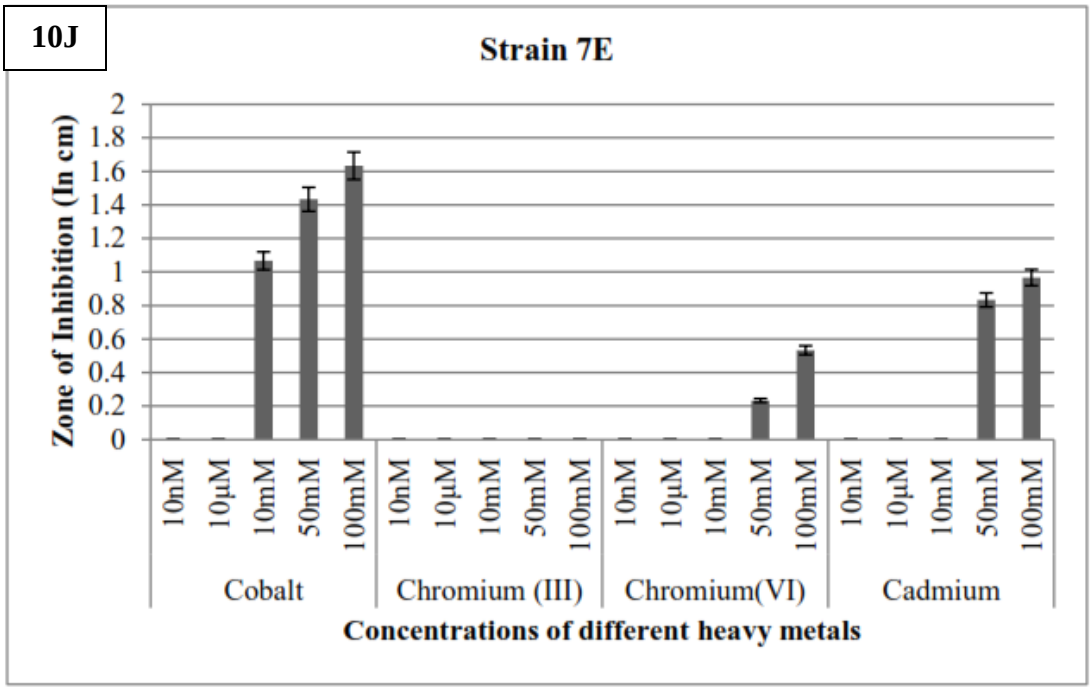
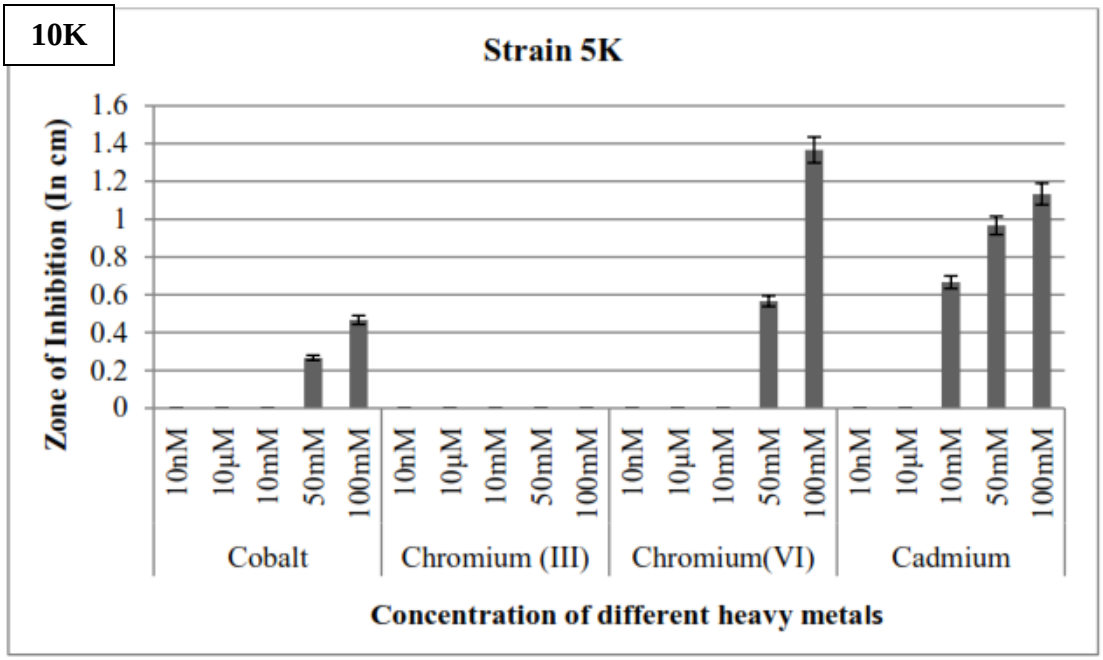
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals

10J

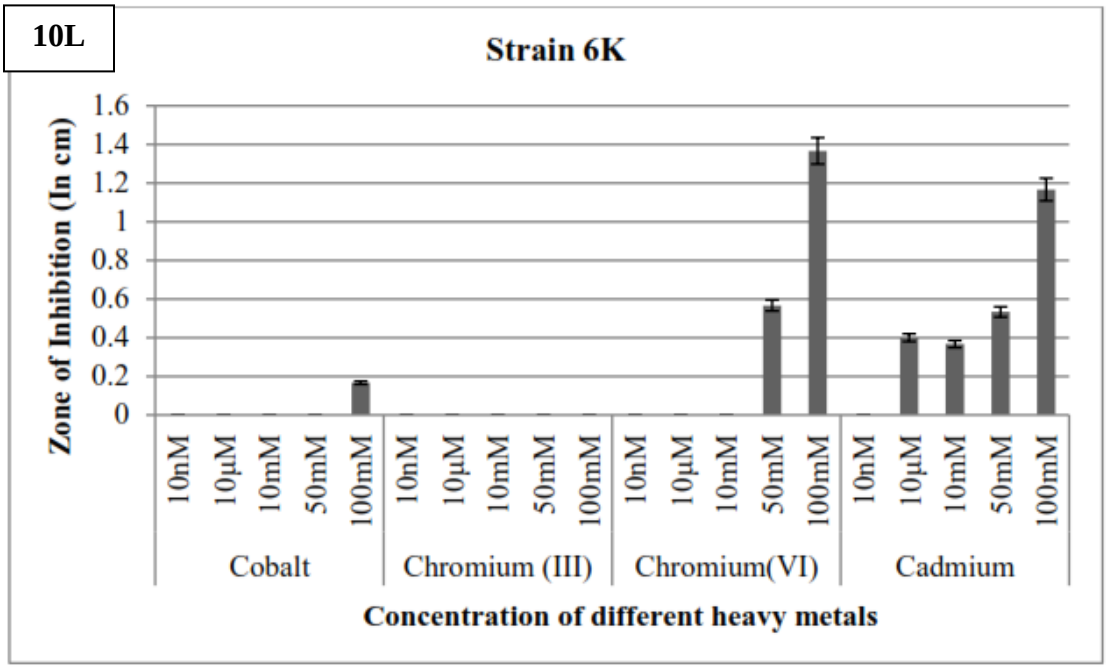

Strain 7E
Zone of Inhibition (In cm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals

III. Bacteria isolated from Kestopur Khal

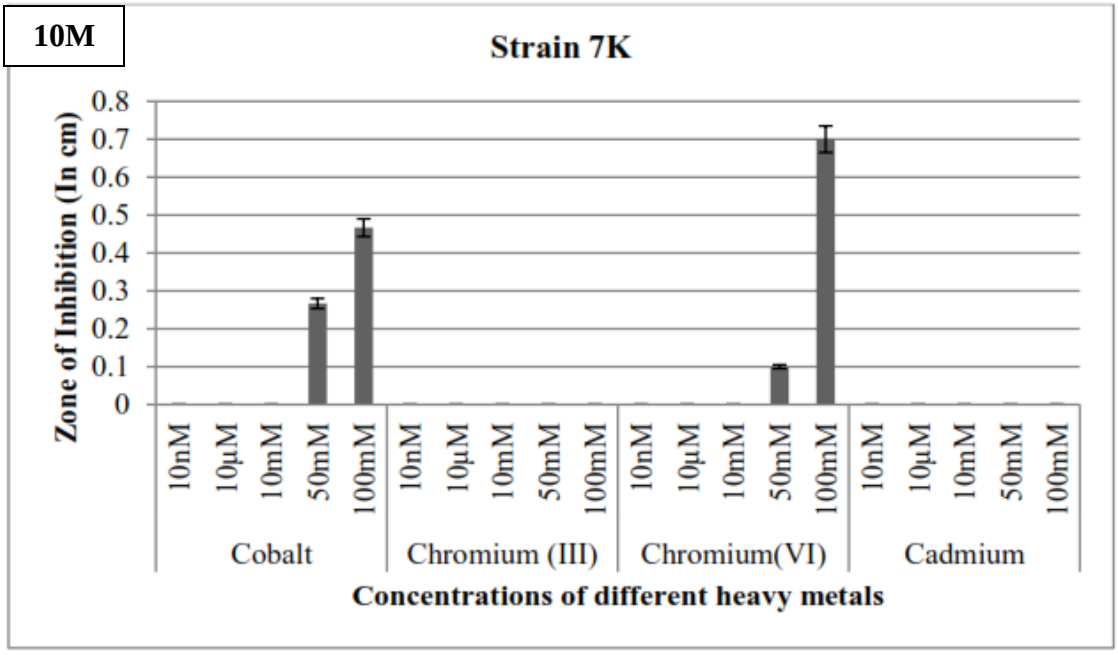
10K



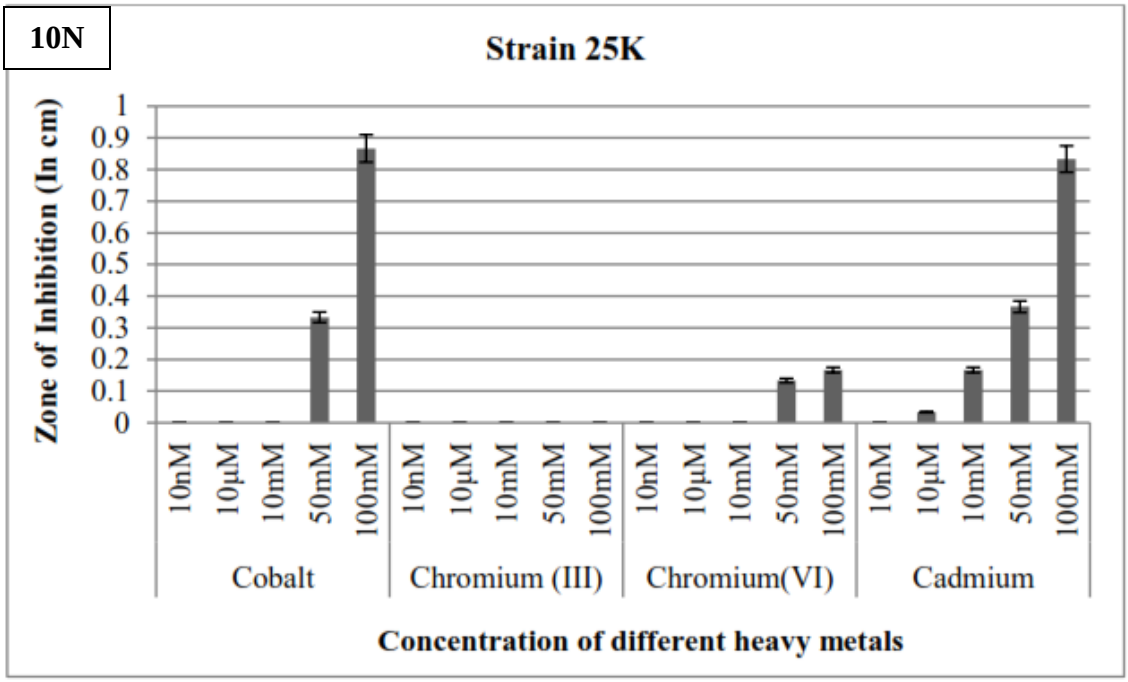
10L



10M


Strain 7K
Zone of Inhibition (In cm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals

10N


Strain 25K
Zone of Inhibition (In cm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals

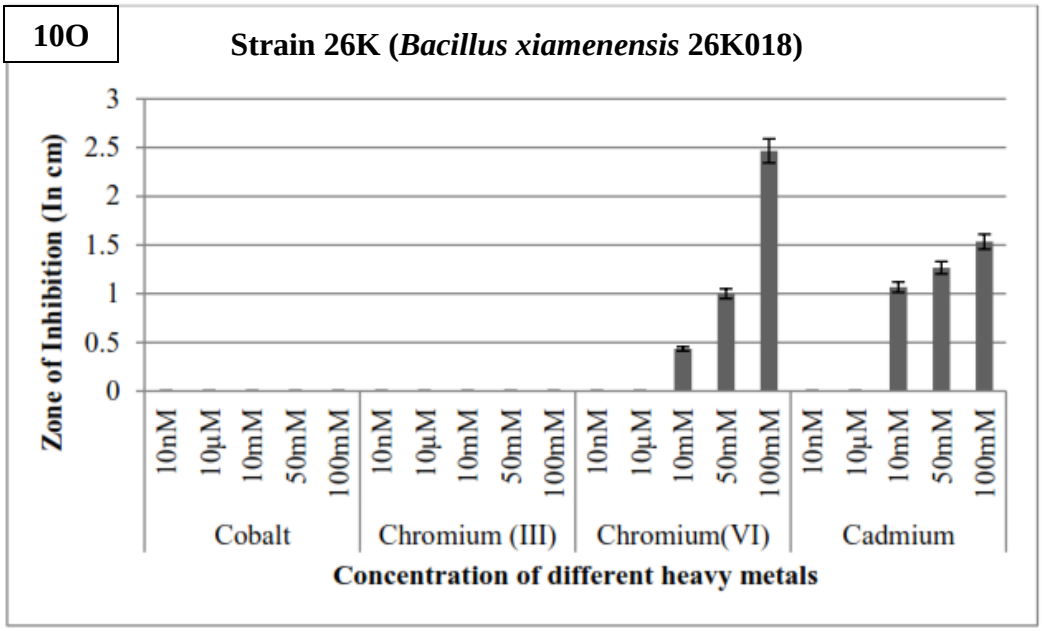


Figure 10 (A-O): Degree of heavy metal sensitivity (in terms of zone of inhibition) of soil microbes as measured by the agar cup method (all the values were obtained by calculating the bacterial zone of inhibition in triplicate)

Interestingly, isolate 12C (later identified to be as *Microbacterium radiodurans* K12016), isolated from Circular canal, shown total resistance against trivalent chromium (Cr^{3+}) till the maximum used concentration (100 mM), both individually and in consortia with other susceptible strains (Figure 11). The Minimal Inhibitory Concentration (MIC) value for hexavalent chromium against *Microbacterium radiodurans* K12016 is 42±0.1 mM. It can therefore be inferred that the chromium resistant *Microbacterium radiodurans* K12016 can be a potent candidate for chromium bioremediation. Two microbial strains (later identified to be as *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 respectively) isolated from East Kolkata Wetland and Kestopur Khal (denoted as 1E and 26K primarily) shown resistance up to 100 mM trivalent chromium. The MIC of hexavalent chromium against *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 was 20±0.19 mM and 10.5±0.1 mM respectively. In spite of low MIC, *Bacillus xiamenensis* strain 1E0018 and 26K018 could resist 100 mM hexavalent chromium in consortia with other susceptible strains.

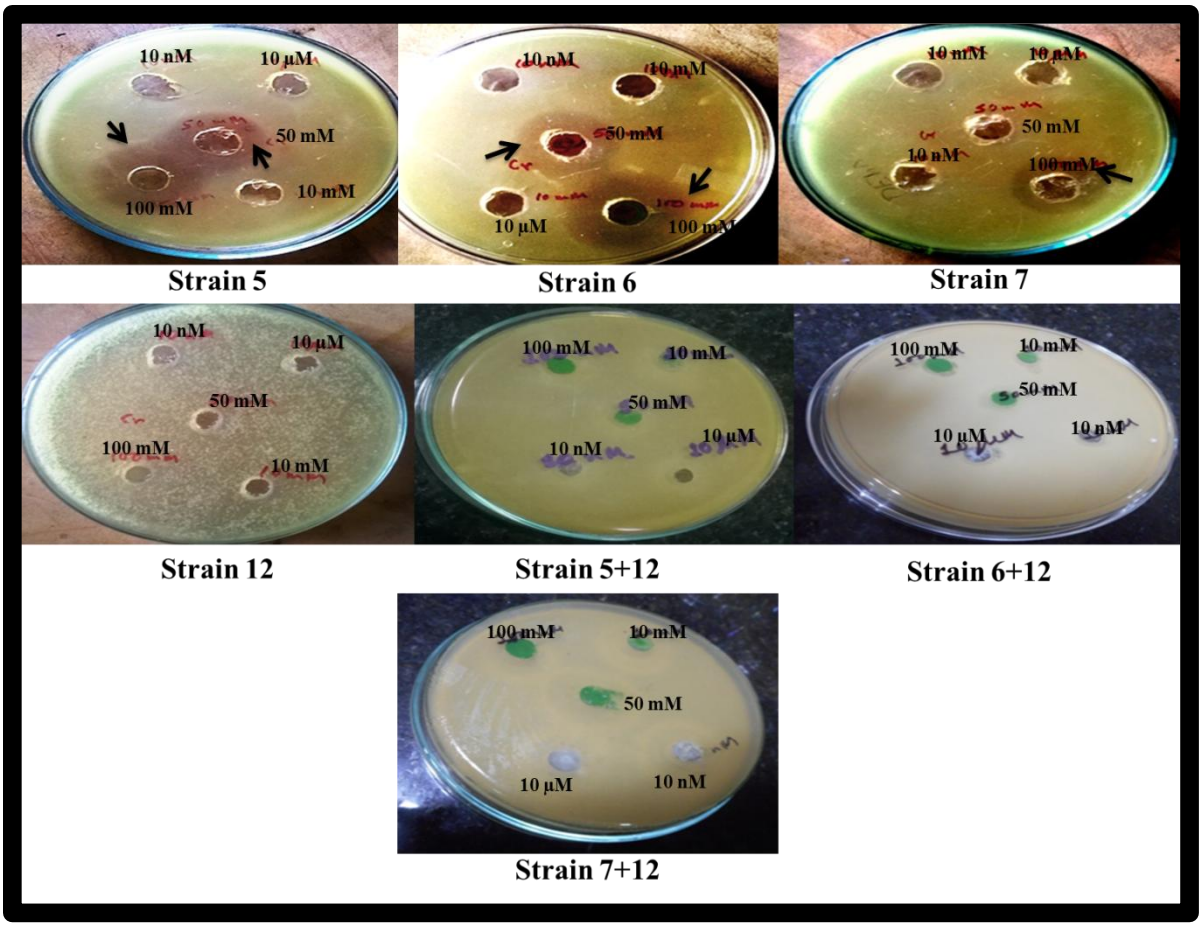


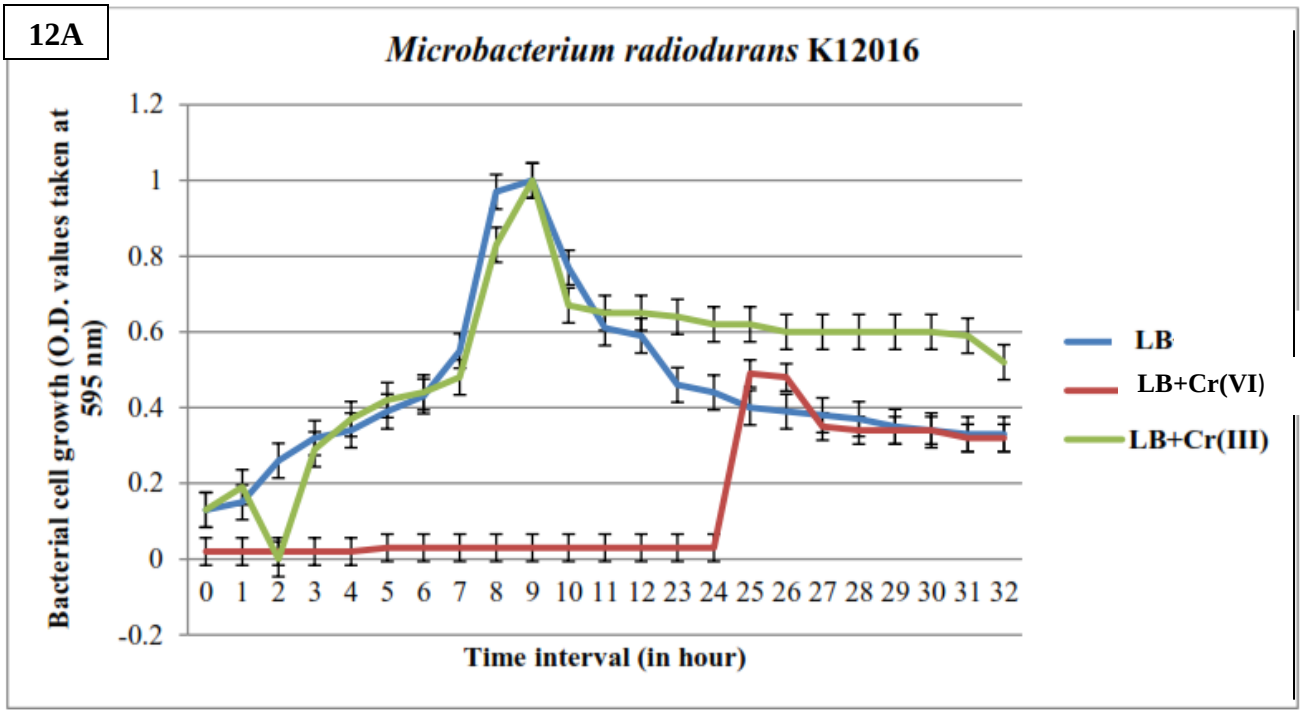
Figure 11: Chromium resistance pattern of microbial isolates 5C, 6C, 7C and 12C (*Microbacterium radiodurans* K12016) respectively, where 12C shown total chromium resistances till the maximum used concentration (100mM). Consortia of strain 12C with susceptible strains 5C, 6C and 7C shown total resistance against 100 mM chromium (The black arrows indicate zones of inhibition)

Three [12C (*Microbacterium radiodurans* K12016), 1E (*Bacillus xiamenensis* 1E0018) and 26K (*Bacillus Xiamenensis* 26K018)] amongst the fifteen selected microbial strains were considerably tolerant to both hexavalent and trivalent chromium (Table 10, Figure 10). These three strains were selected for further experimentations and their probable mechanism of action against carcinogenic hexavalent chromium was investigated.

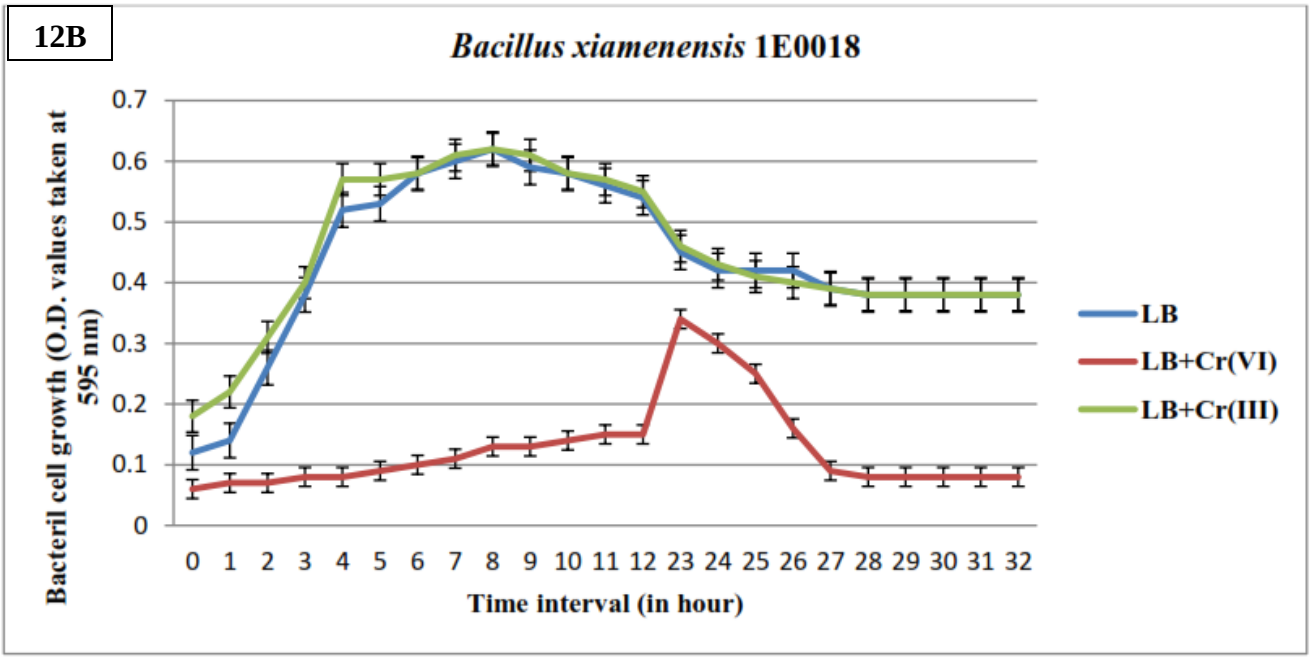
The result of growth profiling in presence and absence of chromium shown that *Microbacterium radiodurans* K12016 exhibited an extended preparatory phase in order to possibly survive in the presence of hexavalent chromium (Figure 12A), thus supporting the observation by Nesheli and coworkers in 2018, which stated that a higher concentration of hexavalent chromium affects the

bacterial growth curve by lengthening the lag phase. *Bacillus xiamenensis* 1E0018 and *Bacillus Xiamenensis* 26K018 show reduced growth rate in the presence of hexavalent chromium (Figure 12B and 12C). However, the bacteria show a normal growth pattern in the presence of trivalent chromium as observed in the metal free environment.

12A



12B



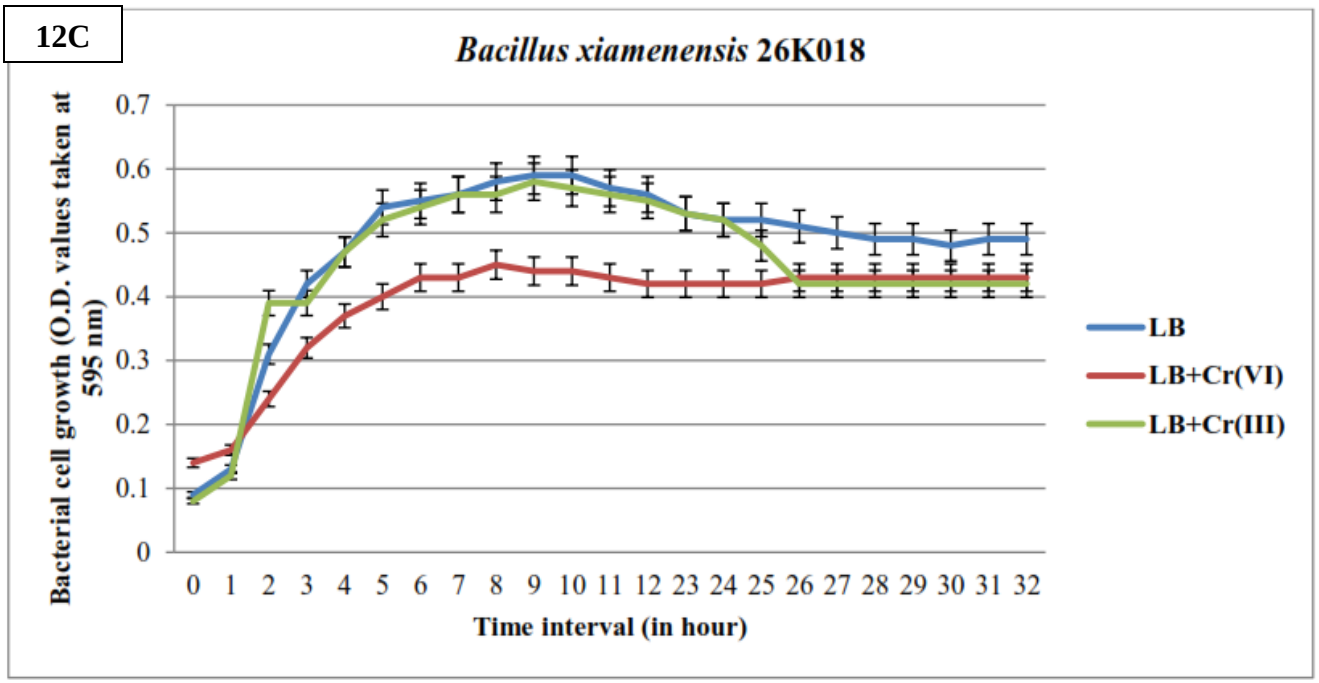
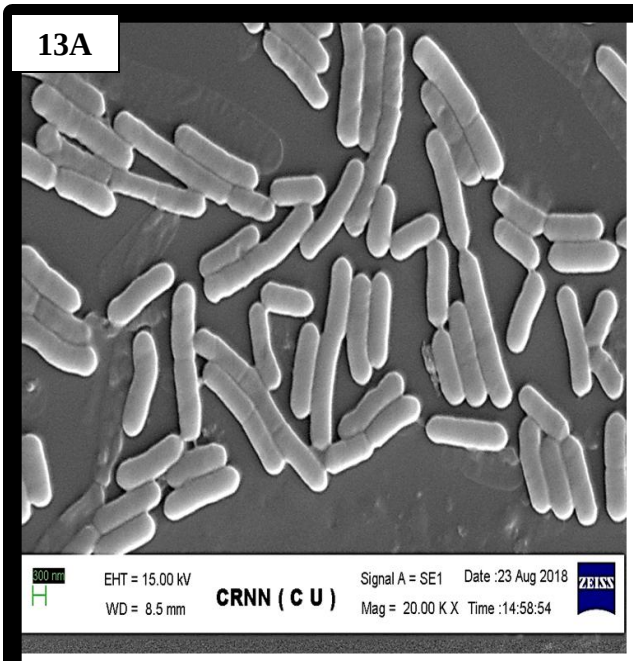


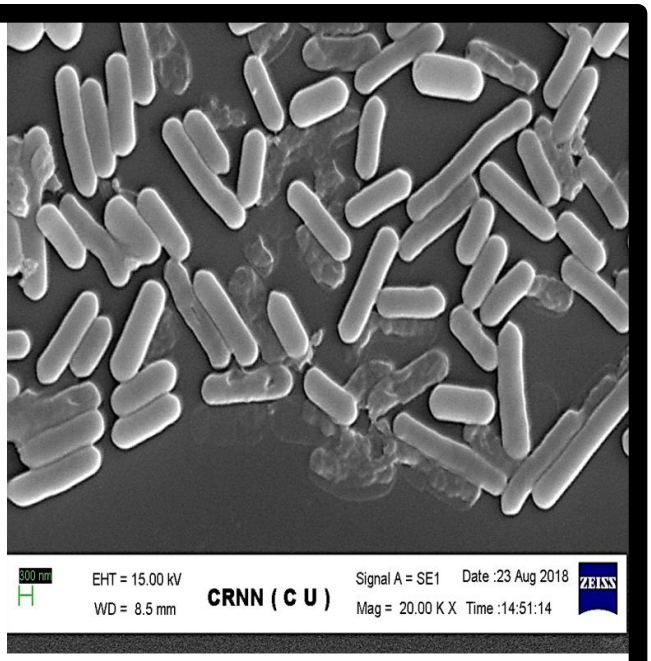
Figure 12 (A-C). **The growth curve of [A] *Microbacterium radiodurans* K12016; [B] *Bacillus xiamenensis* 1E0018; [C] *Bacillus xiamenensis* 26K018 in presence and absence of hexavalent and trivalent chromium**

The SEM micrographs show that 10mM hexavalent chromium-treated cells remained healthy and viable (as the cell surface was found to be unaffected; Figure 13A, 13B, 13C) and became elongated in the presence of chromium to combat chromium-induced stress in comparison to that of the cells grown in chromium-free medium (Figure 13A, 13B and 13C; Table 10). But the 40 mM hexavelent chromium treated cells could not survive because of metal stress (Figure 13D). The results of MIC value measurement also support the above mentioned findings which revealed that 50 mM hexavalent chromium is toxic enough for most of the chromium tolerant microbial isolates. The cells of *Microbacterium radiodurans* K12016 didn't change much morphologically in presence of 10 mM Cr (VI). But the cells grow in 40 mM metal supplemented media which is close to their MIC value (42±0.1 mM), shown distorted cell wall due to cell damage.

13A

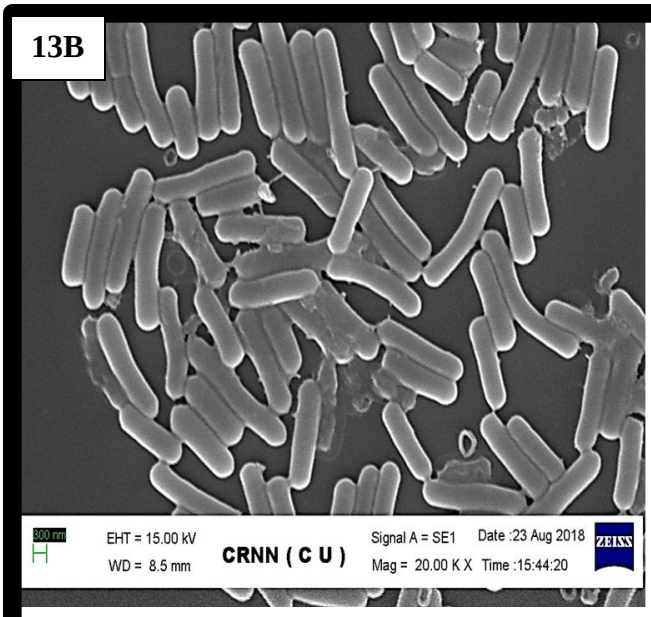


Microbacterium radiodurans **K12016 cell grown in Cr (VI) free medium**

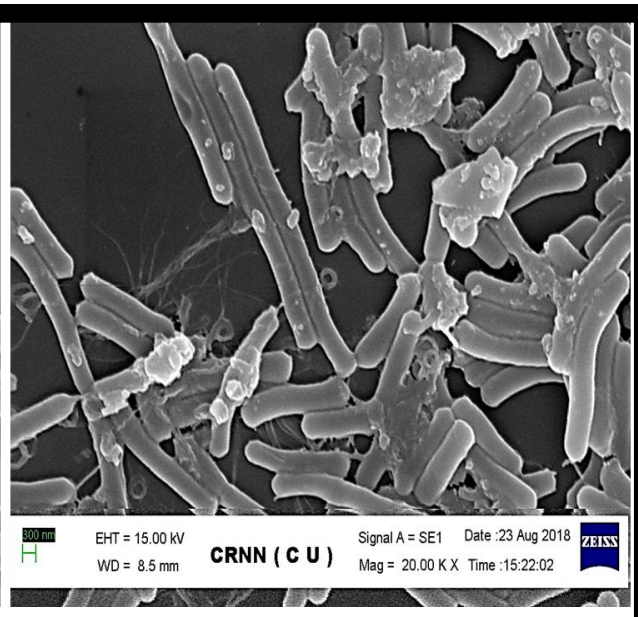


Microbacterium radiodurans **K12016 cell grown in 10 mM Cr (VI) supplemented medium**

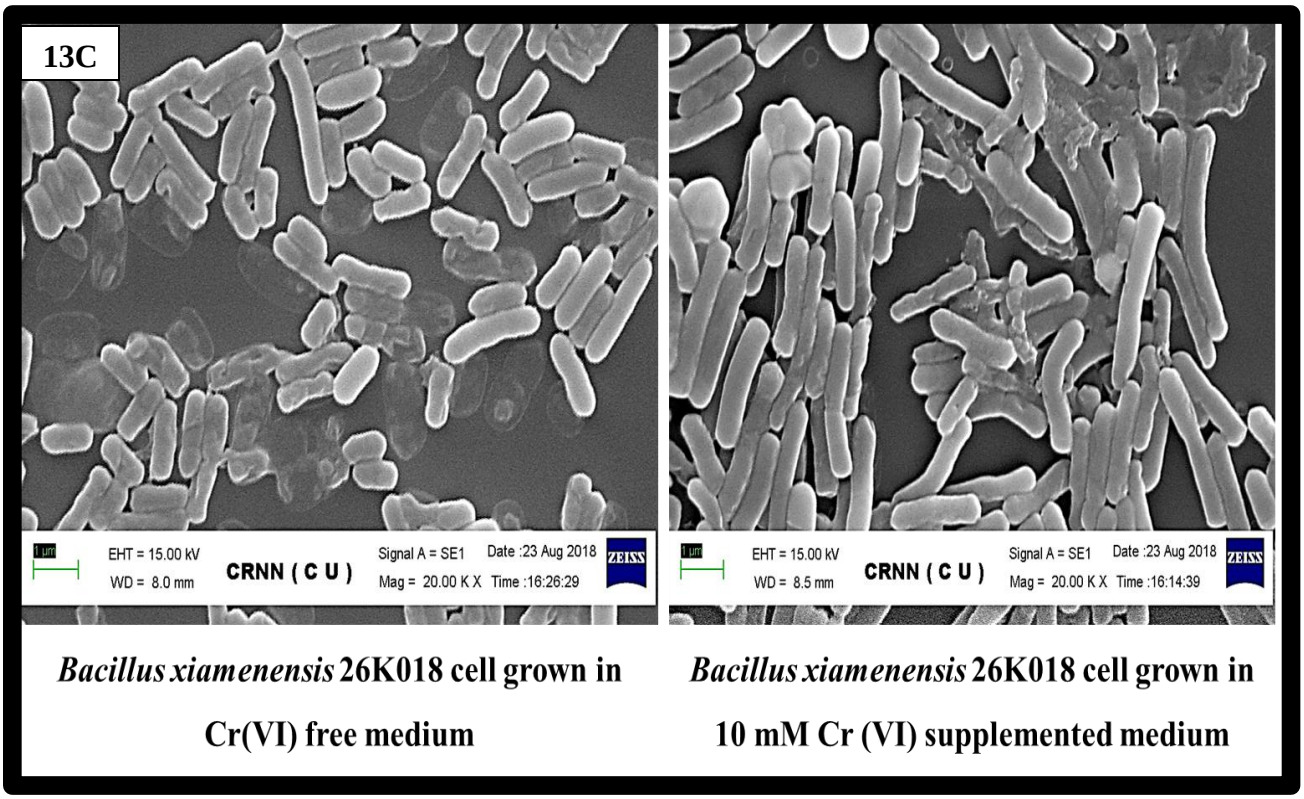
13B



Bacillus xiamenensis **1E0018 cell grown in Cr (VI) free medium**



Bacillus xiamenensis **1E0018 cell grown in 10 mM Cr (VI) supplemented medium**



***Bacillus xiamenensis* 26K018 cell grown in Cr(VI) free medium**

***Bacillus xiamenensis* 26K018 cell grown in 10 mM Cr (VI) supplemented medium**

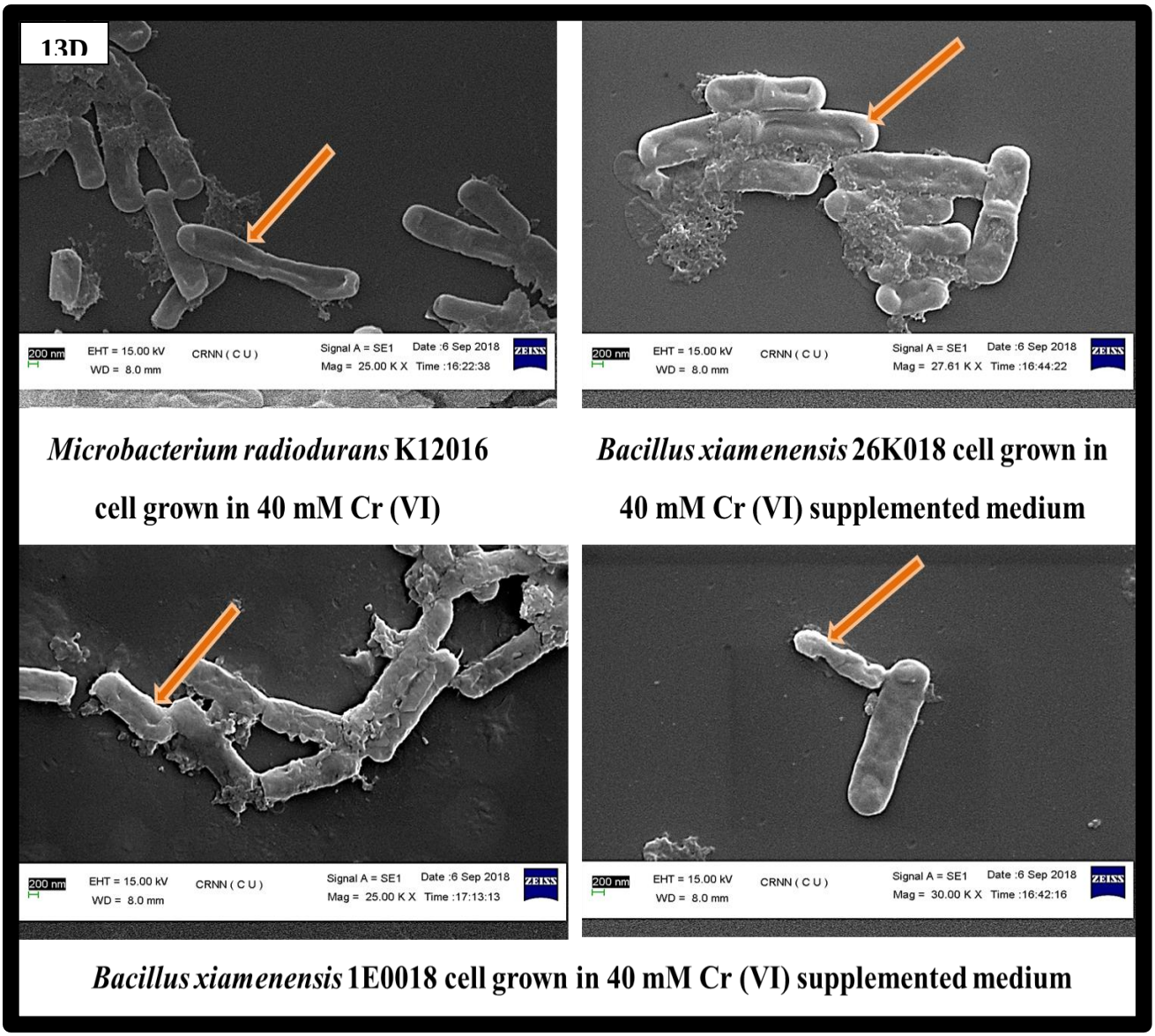

13D
200 nm EHT = 15.00 kV WD = 8.0 mm CRNN (C U) Signal A = SE1 Date :6 Sep 2018 Mag = 25.00 K X Time :16:22:38 ZEISS
Microbacterium radiodurans K12016 cell grown in 40 mM Cr (VI)
200 nm EHT = 15.00 kV WD = 8.0 mm CRNN (C U) Signal A = SE1 Date :6 Sep 2018 Mag = 27.61 K X Time :16:44:22 ZEISS
Bacillus xiamenensis 26K018 cell grown in 40 mM Cr (VI) supplemented medium
200 nm EHT = 15.00 kV WD = 8.0 mm CRNN (C U) Signal A = SE1 Date :6 Sep 2018 Mag = 25.00 K X Time :17:13:13 ZEISS
200 nm EHT = 15.00 kV WD = 8.0 mm CRNN (C U) Signal A = SE1 Date :6 Sep 2018 Mag = 30.00 K X Time :16:42:16 ZEISS
Bacillus xiamenensis 1E0018 cell grown in 40 mM Cr (VI) supplemented medium

Figure 13(A-D): The SEM micrographs (10,000X magnified); Both untreated and 10 mM Cr^{6+} treated cell of [A] *Microbacterium radiodurans* K12016, [B] *Bacillus xiamenensis* 1E0018, [C] *Bacillus xiamenensis* 26K018 remained unaffected and viable; [D] The 40 mM Cr^{6+} treated bacterial cells became distorted and the arrows point out the cell surface distortion due to chromium stress

Table 10: Comparison of length of 10 mM Cr^{6+} and untreated bacterial cell observed under SEM (measured using ImageJ)

Microbial strain	Length of cell grown without metal (nm)	Length of cell treated with 10 mM Cr^{6+} (nm)	P value (calculated by two tailed t-test)	Number of cell studied
Microbacterium radiodurans K12016	910±0.5	1500±0.05	0.0071	10
Bacillus xiamenensis 1E0018	1342±0.6	3180±1.1	0.0006	10
Bacillus xiamenensis 26K018	1270±0.7	2950±2.9	0.0002	10

Chromium removal strategies implemented by isolated soil microbes:

The chromium removal efficiencies of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 were investigated against carcinogenic hexavalent chromium.

Microbacterium radiodurans K12016 cultured in 51.9 ppm (10 mM) and 259.5 ppm (50 mM) hexavalent chromium supplemented medium, absorbed 35.73 ± 0.21ppm and 86.7±0.6 ppm metal, respectively, after 24 hours of incubation. The 1E0018 and 26K018 strains of *Bacillus xiamenensis*, absorbed 40.61±0.86 ppm and 41.53±1.27 ppm chromium from the 51.9 ppm (10 mM) metal supplemented media after 24 hours of incubation. All the data were calculated from three repeated experiments (Table 11).

The percentage of removal and amount of metal absorption per unit of bio absorbent was calculated using formulae:

a) Percentage of removal = (Initial concentration - Final concentration/Initial concentration) × 100

b) Metal absorption per unit of bio absorbent = (Initial concentration - Final concentration/ Mass of bio absorbent) × Concentration of metal solution

Table 11: Bacterial Cr(VI) absorbance and absorbed metal removal:

Microbial isolates	**Concentration of Cr in growth media before culture (ppm)**	**Cr available in growth media (ppm) after 24 hours of microbial growth (ppm)**	**Cr absorbed by microbe from growth media (ppm)**	**Cr released back by microbe to metal free media (ppm)**
***Microbacterium radiodurans* K12016**	51.9	15.73 ± 0.19	35.73 ± 0.21	0.000129±0.20
***Microbacterium radiodurans* K12016**	259.5	173.7±0.53	86.7±0.6	0.000325 ± 0.39
***Bacillus xiamenensis* 1E0018**	51.9	11.28±0.7	40.61±0.86	<0.00002 (negligible)
***Bacillus xiamenensis* 26K018**	51.9	9.96±1.9	41.53±1.27	0.00048 ± 0.25

The results from Table 12 stated that a considerable amount of chromium from the surrounding (culture media) has accumulated in the bacteria and released back a negligible amount of accumulated chromium in the metal free media (Table 13). These findings indicate that the isolated microbes can be used as well bioremediating agent because of their chromium absorbance and nominal release of the absorbed chromium from bacteria.

Table 12: Chromium absorption (i.e. removal from surrounding media) from per mg of bacterial biomass:

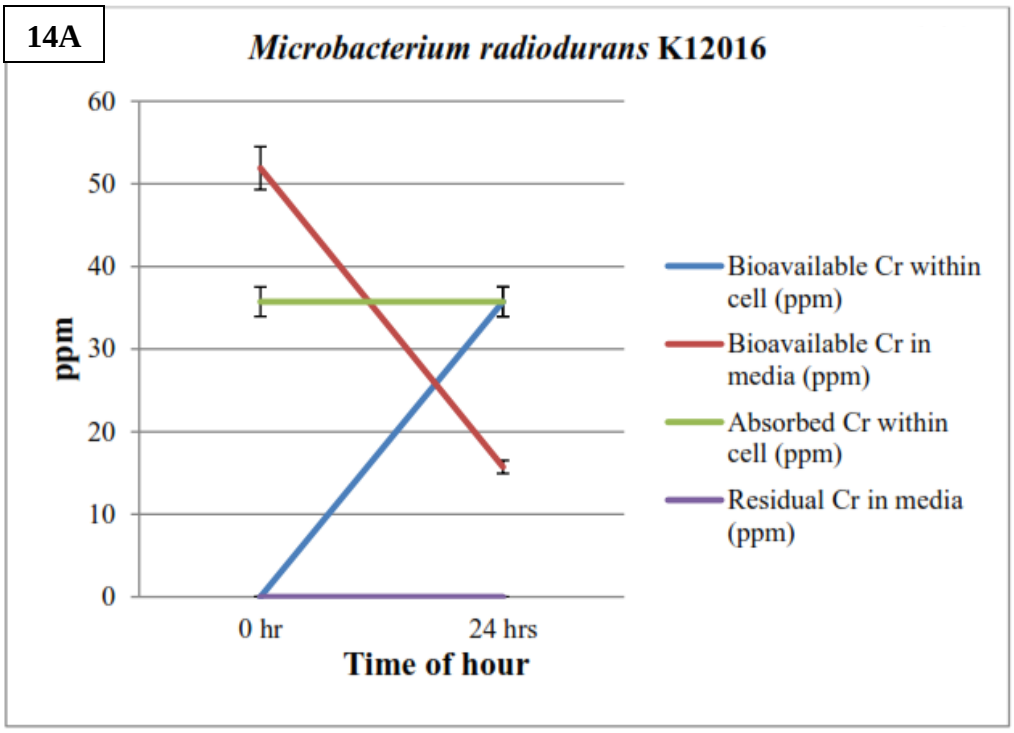
***Microbacterium radiodurans* K12016 (ppm/mg/hr)**		***Bacillus xiamenensis* 1E0018 (ppm/mg/hr)**	***Bacillus xiamenensis* 26K018 (ppm/mg/hr)**
Grown in 10 mM Cr supplemented media	***Grown in 40 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***
1.37±0.78	**4.59±0.97**	**12.9±0.92**	**1.86±0.09**

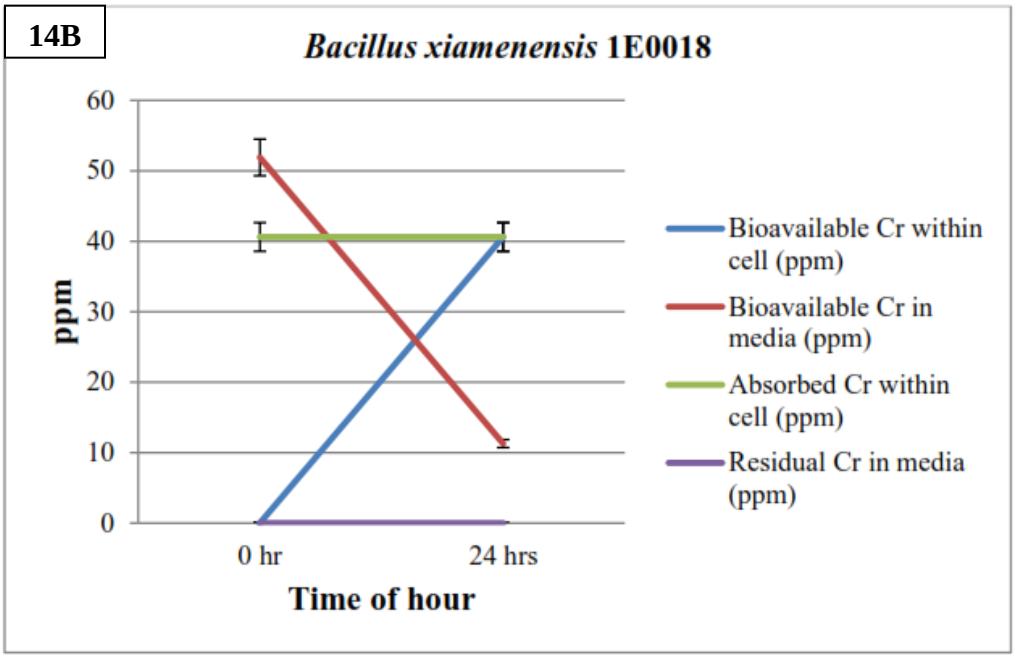
Table 13: Percentage of chromium released in metal free media from chromium accumulated bacterial biomass (in 24 hours):

***Microbacterium radiodurans* K12016 (Percent)**		***Bacillus xiamenensis* 1E0018 (Percent)**	***Bacillus xiamenensis* 26K018 (Percent)**
Grown in 10 mM Cr supplemented media	***Grown in 40 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***
0.45±0.1	**0.72±0.002**	**Negligible**	**0.053±0.05**

The bacterial cell released back only a negligible amount of the absorbed heavy metal to the metal free media after three serial batch cultures. *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 cell absorbed the metal from hexavalent chromium supplemented media, released only 0.000325 ± 0.39 mg/l, <0.00002 mg/ml (negligible) and 0.00048 ± 0.25 mg/l of chromium to the metal free media respectively (Table 14).

Bioavailable chromium present in the media was decreased after 24 hours for *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 bacterial cells whereas the bioavailable chromium contents within these bacterial cells were increased after 24 hours of incubation. The bacterial accumulated chromium retained within the cell and no considerable amount of chromium was released out in the metal free media (Figure 14).





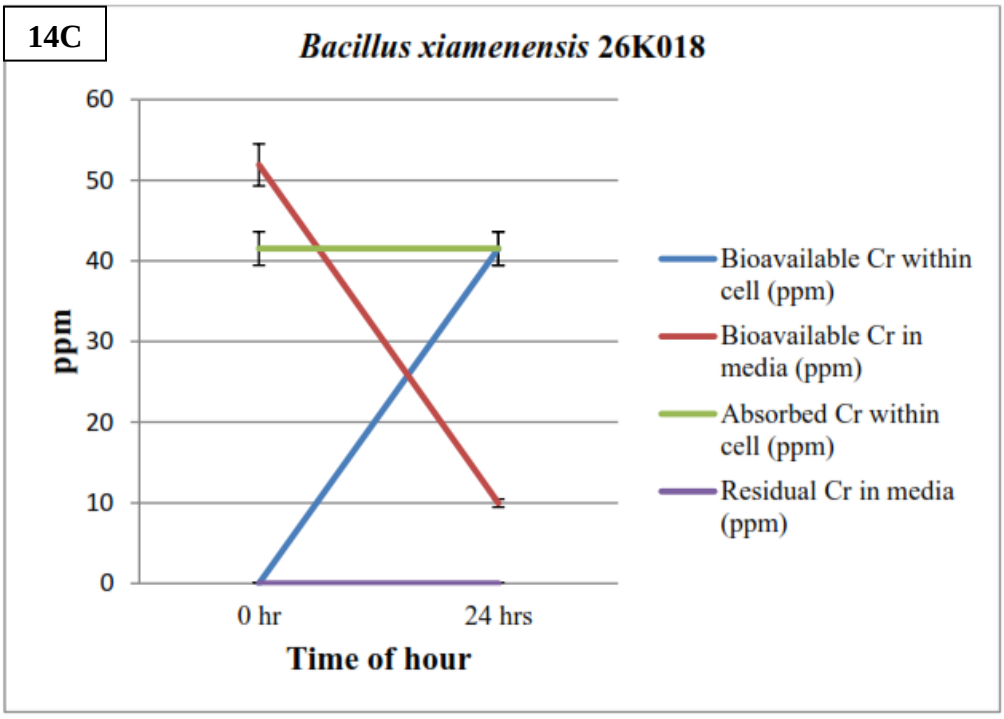
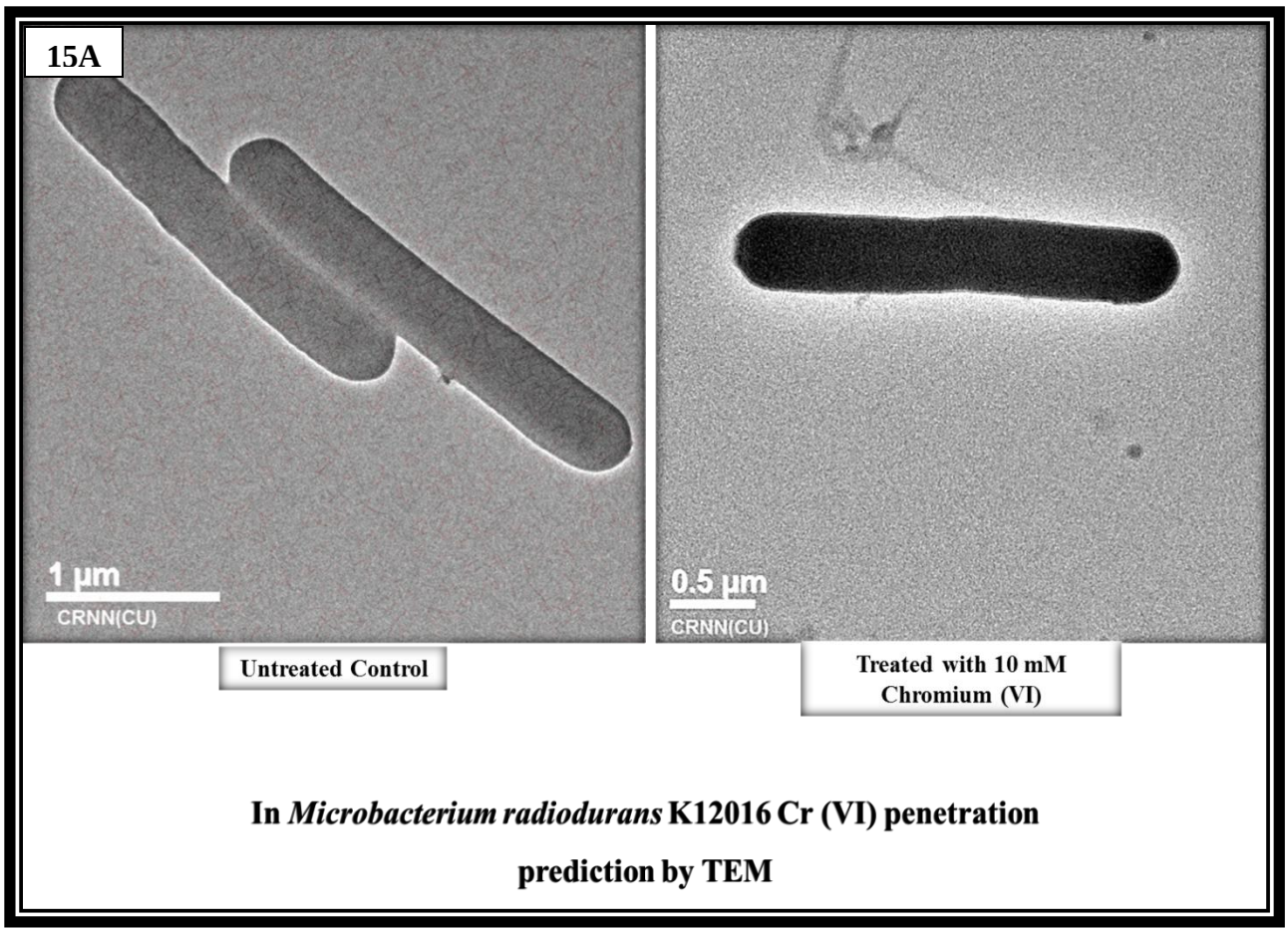
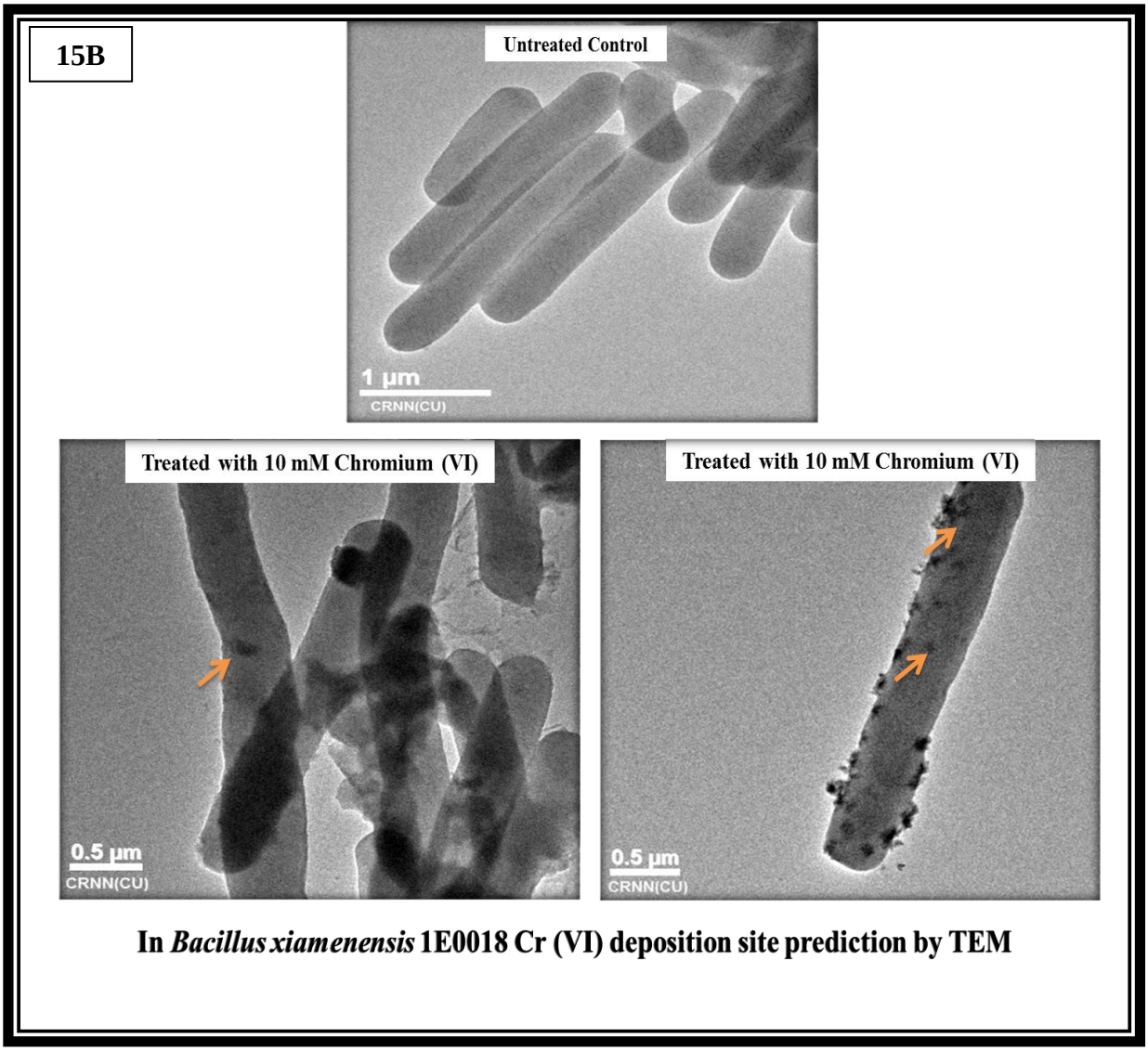


Figure 14 (A-C): Comparison between the chromium absorption and removal efficiency selected microbial isolates after 24 hour incubation

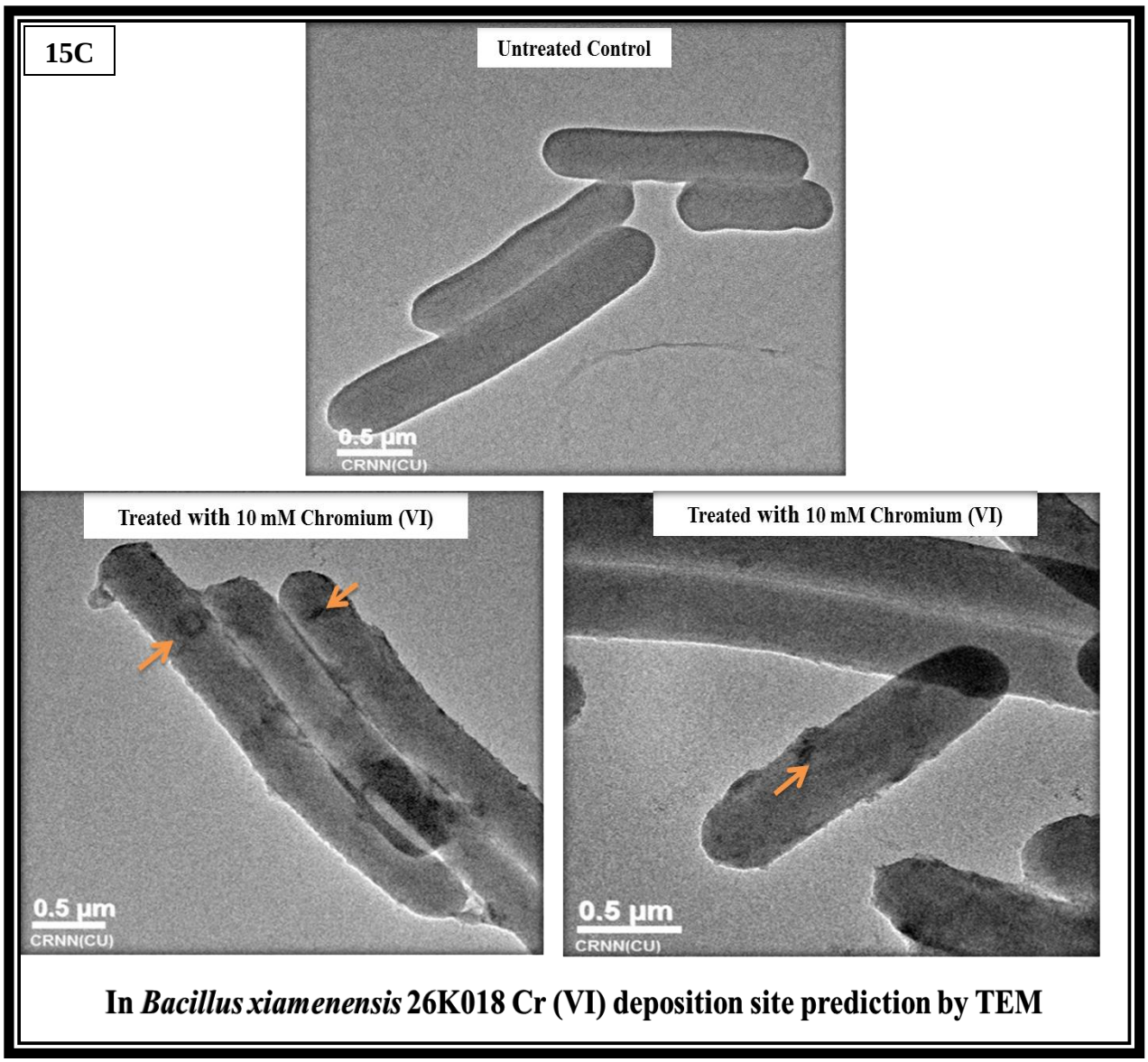
Chromium penetration and deposition within the chromium treated microbial cell was predicted by observing the TEM micrographs (T.M. Roane, 1999). The TEM micrographs show dark patches only in the presence of heavy metals. The cell which was in chromium free environment, didn't show any dark patch inside the cell, but the cell treated with chromium, were shown dark patches. An extensive chromium penetration was observed within the cells of *Microbacterium radiodurans* K12016 which may be due to positive staining by chromium (Figure15A). Dark patches were observed inside the chromium treated cell of *Bacillus xiamenensis* 1E0018 (Figure 15B) and *Bacillus xiamenensis* 26K018 [Figure 15C] but no patches were found in control cells (grown in chromium free media) which may be the indication of presence of chromium deposition inside cell. Dark patches were also observed at the cell surface of *Bacillus xiamenensis* due to the presence of chromium.



In *Microbacterium radiodurans* K12016 Cr (VI) penetration prediction by TEM



In *Bacillus xiamenensis* 1E0018 Cr (VI) deposition site prediction by TEM



In *Bacillus xiamenensis* 26K018 Cr (VI) deposition site prediction by TEM

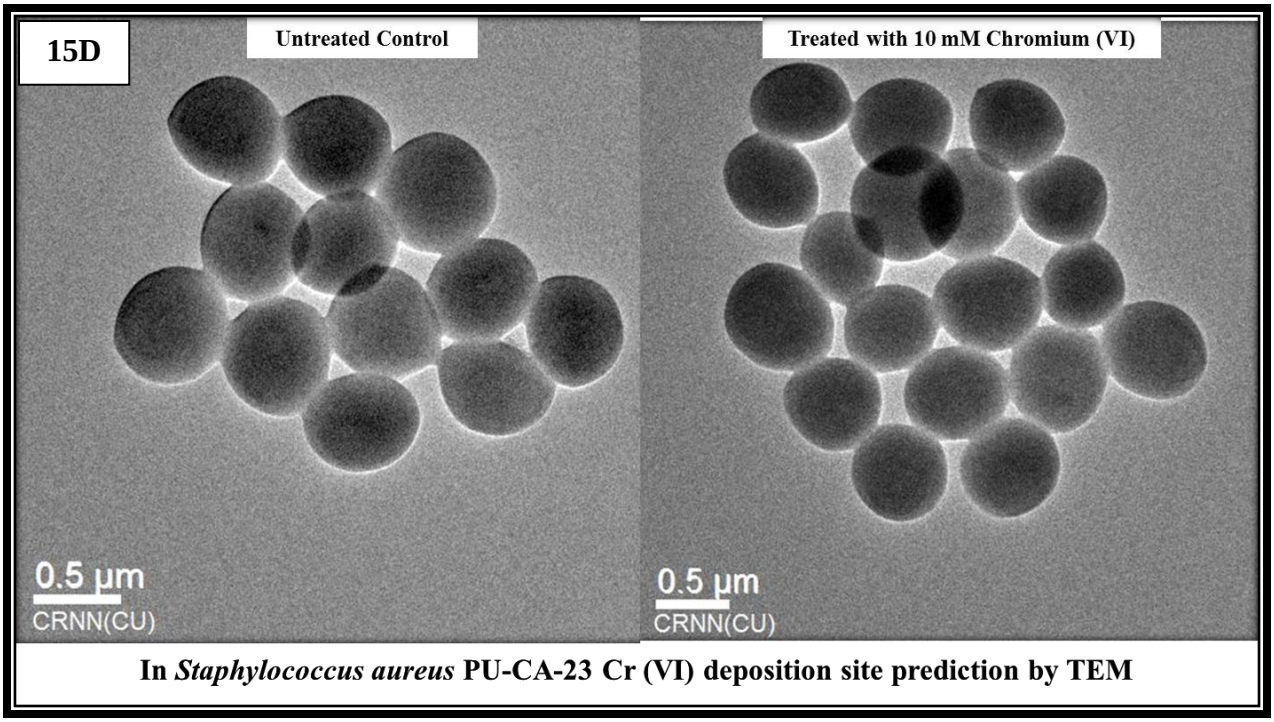


Figure 15(A-C): Prediction of chromium penetration and deposition within 24 hours treated bacterial cells by TEM micrographs. The arrows indicate chromium deposition sites. Figure 15D: The Cr(VI) treated cells of *Staphylococcus aureus* PU-CA-23 used as negative control where the bacteria were not influenced by hexavalent chromium and no metal deposition was found within cells

It can be stated from the chromium absorbance and removal assay and TEM micrographs that bacteria cultured in hexavalent chromium-supplemented media absorbed hexavalent chromium. However, Diphenylcarbazile spectrophotometry (APHA 23rd Ed.) confirmed that there were no traces of hexavalent chromium within the cultured bacterial cell pellet. This finding predicted that the toxic hexavalent chromium was converted to less hazardous trivalent chromium by the isolated soil bacteria. The sodium chloride and ammonium hydroxide test also confirmed the presence of trivalent chromium within bacterial cells cultured in Cr(VI) supplemented media.

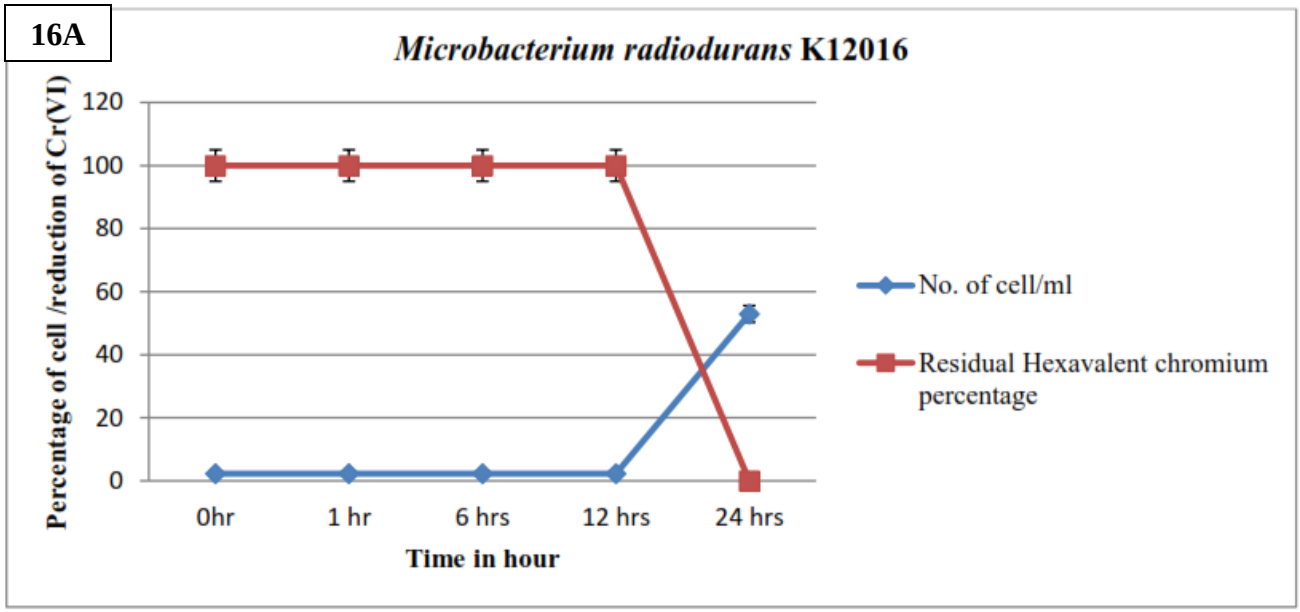
In hexavalent chromium supplemented media, Cr(VI) was reduced gradually during bacterial growth (Figure 16, Figure 17). The microbes of our interest completely reduce 10mM Cr(VI) within 24 hours. *Microbacterium radiodurans* K12016 reduce 65±0.01% of 40 mM Cr(VI) in 24 hour and 100% Cr(VI) in 48 hours.

Percentage of hexavalent chromium reduction was calculated using the formula:

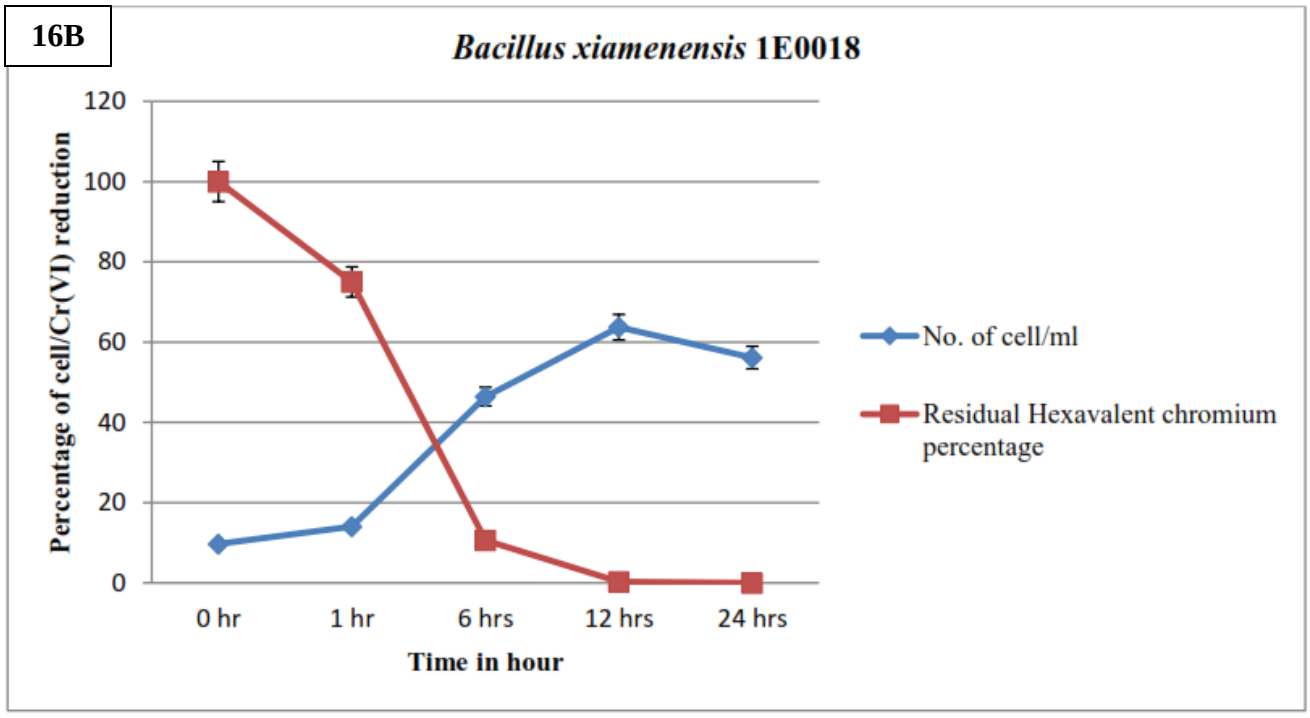
Percentage of hexavalent chromium reduction = [(Absorbance of control - absorbance of chromium treated cell sample)/absorbance of chromium treated cell sample] ×100.

Where cell free hexavalent chromium supplemented media was considered as control.

16A



16B



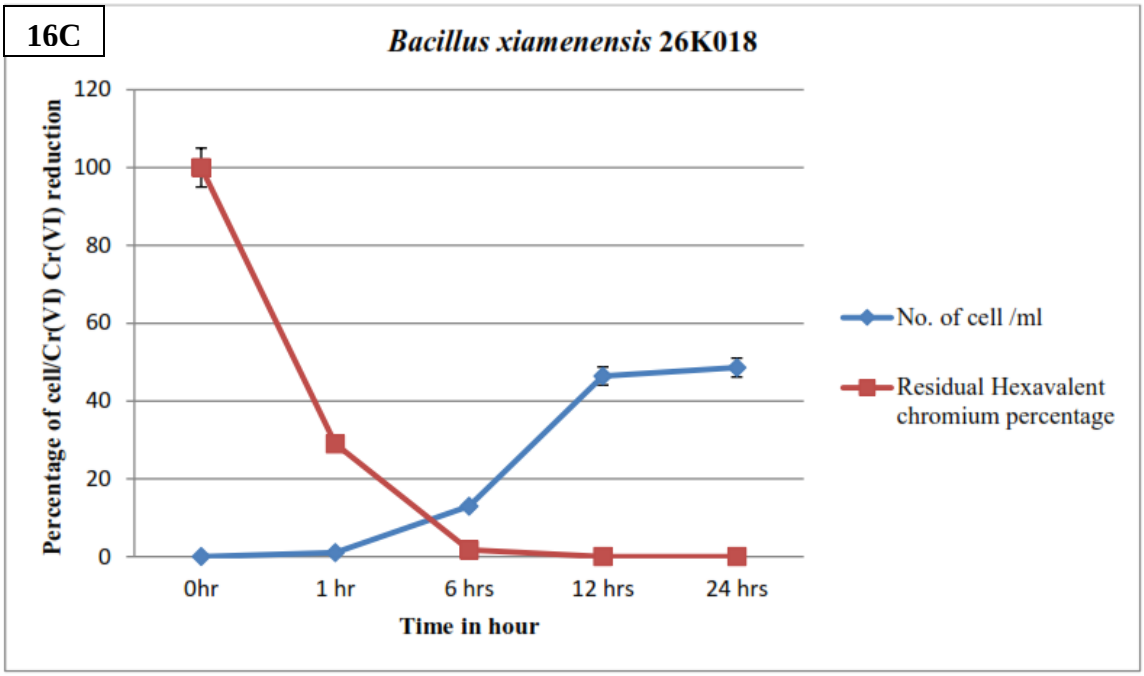


Figure 16(A-C): Hexavalent chromium reduction in viable bacterial cell and the simultaneous growth of bacteria cultured at 10 mM chromium supplemented media at the specific time course

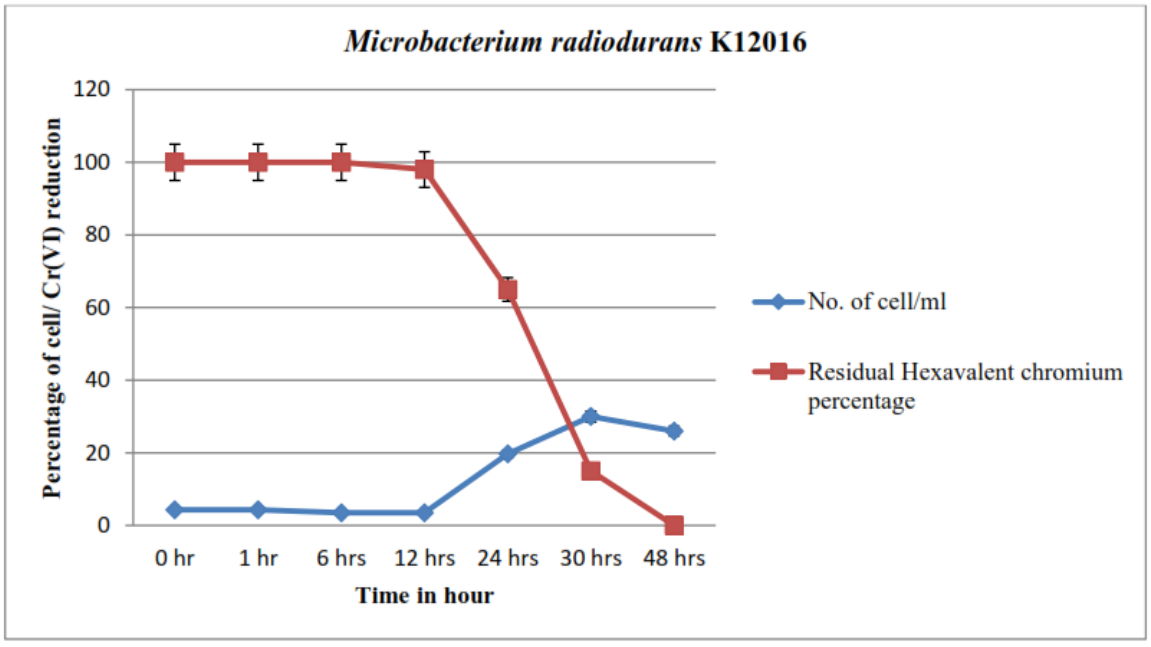


Figure 17: Hexavalent chromium reduction in viable bacterial cell and the simultaneous growth of bacteria cultured at 40 mM chromium supplemented media at the specific time course

When we observed the rate of reduction of hexavalent chromium in presence of microbial growth stimulators sodium azide and DNP, we find that DNP initially accelerated the rate of chromium reduction in *Microbacterium radiodurans* K12016 in comparison to control and sodium azide decelerated it. In case of the *Bacillus xiamenensis* strains (1E0018 and 26K018), DNP didn't show any considerable change in rate of chromium reduction (Figure 18).

Previous study reported that respiratory and microbial growth inhibitor sodium azide inhibits reduction by nullifying the activity of enzyme enolase, cytochrome oxidase, ATPase. Our results also showed similar trends for *Microbacterium radiodurans* K12016 (figure 18). The present study also indicates that, DNP treated cells accelerate the rate of chromium reduction and as we know that DNP accelerate respiratory chain linked electron transport, increase proton permeability of mitochondrial inner membrane and stimulate aerobic respiration and chromium reduction simultaneously (Shen and Wang, 1993), it can be stated that the promoting effect of DNP in *Microbacterium radiodurans* K12016, probably comes from its acceleratory role in respiratory chain of electron transport.

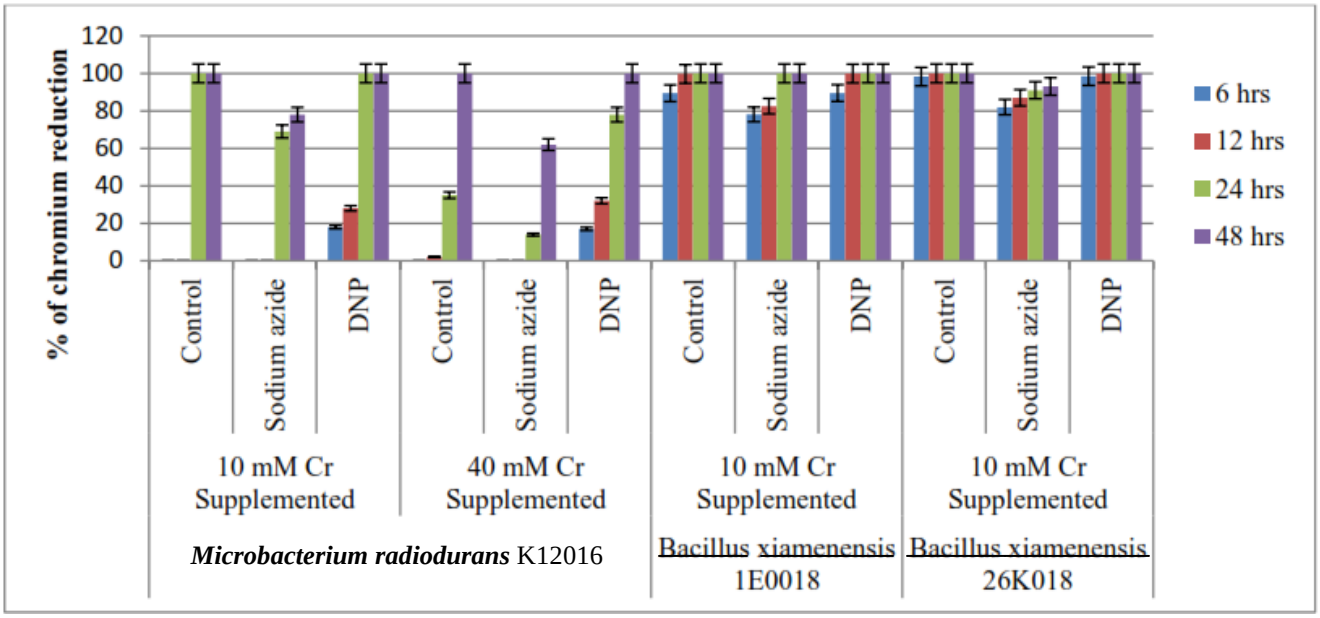


Figure 18: Influence of different microbial stimulators on chromate reduction

The newly identified chromium removing *Microbacterium radiodurans* K12016 was used to carry out megablast alignment with chromium reducing *Microbacterium oleivorans, Microbacterium liquefaciens* and get respectively 90.72% and 87.08% 16S rRNA based sequence identity. *Microbacterium oleivorans* was reported as chromium reducing strain and expressing chromate reductase gene chrA (Sarkar *et al.* 2016). Some *Bacillus* sp. including

Bacillus subtilis (express chromate reducing nfrA gene), *Bacillus cereus*, *Bacillus firmus* exhibited more than 90% sequence similarity with the newly identified *Bacillus xiamenensis*, possess a potential role in the aerobic chromium reduction (Garbisu et al. 1998; Liu *et al.* 2006; Xiao *et al.* 2017; Sau *et al.* 2010; Zheng *et al.* 2014). As we have already known that in prokaryotes, 16S rRNA control the function of mRNA through small ribosomal sub-unit (Rinke-Appel *et al*, 1994) and contribute mRNA stabilization because eight out of ten rRNA bases contact mRNA, are >99% conserved in all phylogenetic domains (Abdi and Fedrick, 2005). Our experimental results indicate that the bacteria which are closely related to our identified *strains, Microbacterium radiodurans* K12016, and *Bacillus xiamenensis* 26K018, exhibit some functional similarity.

Objective III: Role of rhizospheric microbe in decreasing plant heavy metal accumulation:

Plant Growth Promoting Rhizospheric (PGPR) traits of isolated soil microbe:

The plant rhizospheric bacteria promote plant growth because they produce different extracellular plant growth stimulating substances such as Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), siderophore, and phosphate degrading enzymes in the plant rhizospheric soil. The Indole compounds are synthesized by plants, fungi and a few microbes and play key role in root and shoot development for its centrifugal movement and also help in plant cell division and elongation. Most of our isolated soil bacteria also produced Indole compounds (Table 14).

Table 14. Concentration of Indole compounds produced by rhizospheric soil bacteria (implemented using standard curves provided by Sigma-Aldrich). The experiments were repeated in triplicate and the values were calculated with the help of standard solutions (± standard deviation)

Bacterial Strain	Indole compounds formed (in µg) in per ml of surrounding bacterial media
1C	37.5±0.2
2C	25±0.28
3C	37.5±0.11
4C	43.75±0.16
5C	43.75±0.1
6C	37.5±0.2
7C	37.5±0.2
8C	31.25±0.3
9C	37.5±0.05
10C	24.5±0.11
11C	25±0.5

12C (*Microbacterium radiodurans* K12016)	25±0.36
1E(*Bacillus xiamenensis* strain 1E0018)	13±0.06
2E	27±0.12
3E	25±0.24
4E	25±0.09
5E	27±0.07
6E	15±0.3
7E	14±0.08
8E	28±0.24
9E	21±0.09
10E	14±0.24
11E	16±0.32
12E	21±0.06
13E	35±0.21
15E	29±0.04
1K	31±0.2
2K	27±0.7
3K	26±0.28
4K	21±0.09
5K	32±0.3
6K	31±0.09
8K	28±0.04
9K	37±0.07
10K	35±0.32
11K	32±0.34
12K	31±0.09
13K	33±0.32
16K	22±21

18K	33±0.09
25K	29±0.01
26K (*Bacillus xiamenensis* strain 26K018)	39±0.04

The red coloration formed due to addition of Salkowski reagent (0.5M $FeCl_2$ and 35% perchloric acid) to the L-tryptophan supplemented media indicated the presence of Indole compounds (IAA, IBA). (Figure 19).

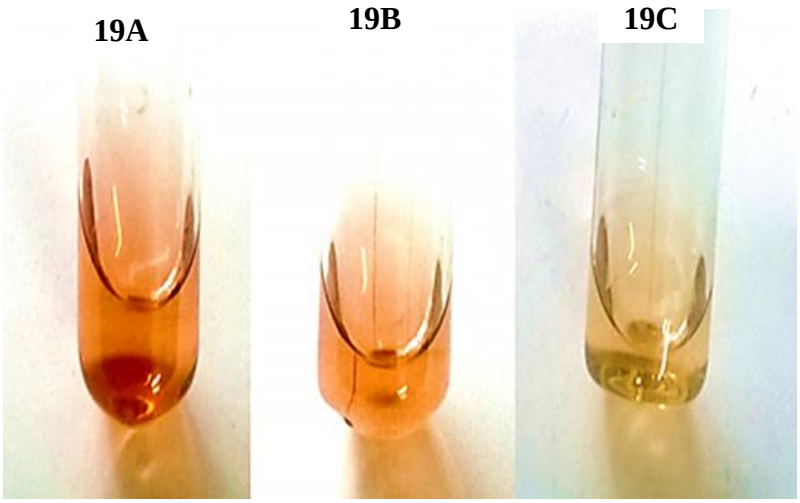


Figure 19 (A-C): The culture tubes shown different gradation of red colour after adding Salkowski reagent to L-tryptophan supplemented cell free bacterial media due to formation of roslindale dye in different concentration by Salkowski reagent and microbial isolate produced Indole compounds from L-tryptophan .

The cell free extracts of some selected microbes were subjected to HPTLC to confirm the presence and concentration of IAA and IBA. The co-chromatogram plots showed the presence of IAA and IBA peaks in all the samples compared with standard solutions (Figure 20).

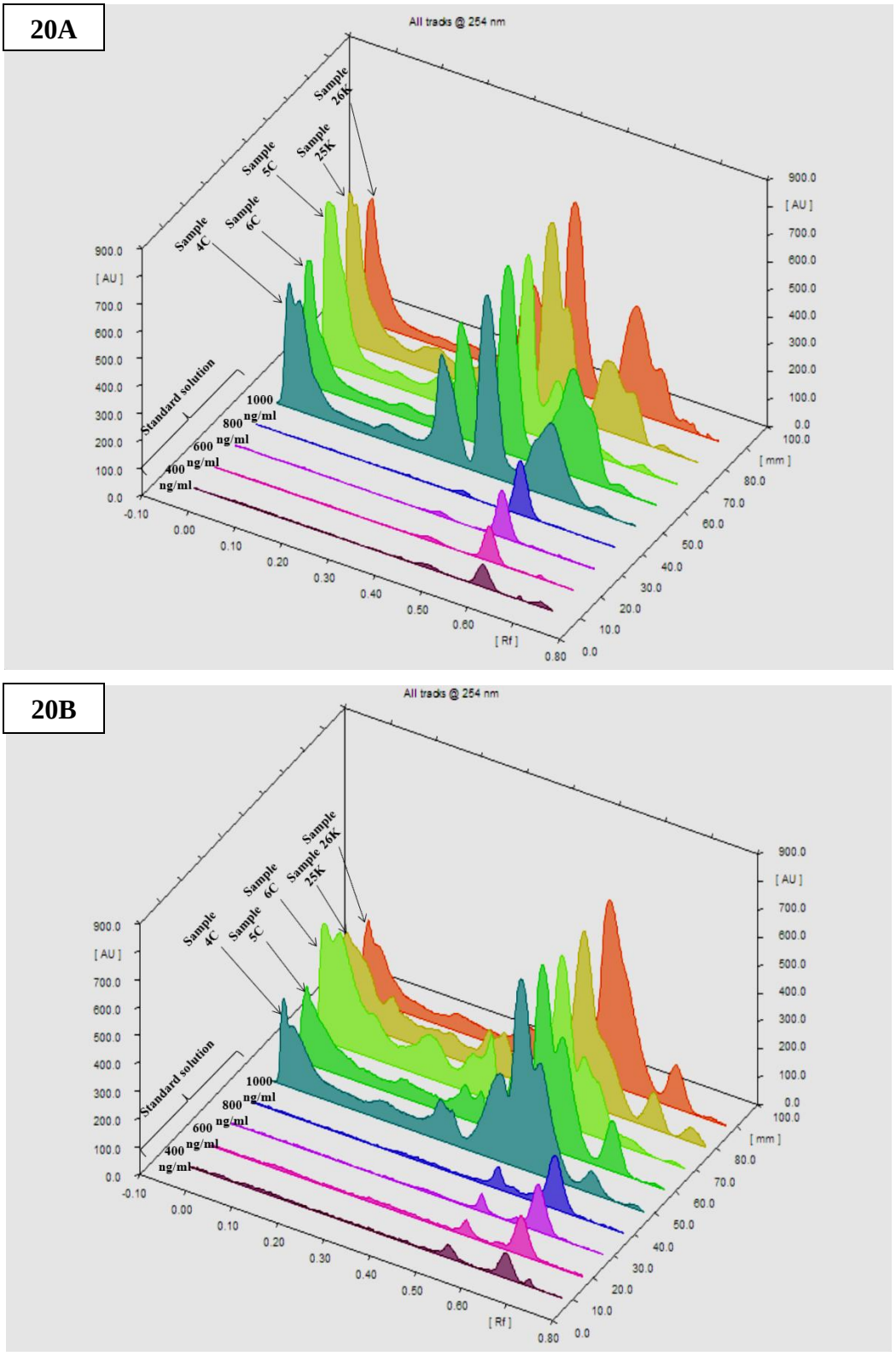


Figure 20: HPTLC chromatogram plot of IAA [A] and IBA [B]

The calibration curves prepared with standard solutions in the range of 400ng/ml, 600ng/ml, 800ng/ml and 1000ng/ml for IAA and IBA are linear. Comparison of the peaks of standard IAA and IBA of known concentrations, revealed the identity of IAA and IBA present in all the samples. Using the standard curve, the concentrations IAA and IBA within our samples was calculated and it can be stated that amongst the selected Indole producing microbe, majority produces IBA (Table 15).

Table 15: Concentration of IAA and IBA produced by Different microbial isolates measured by HPTLC technique:

Strain	IAA (ng/ml)	IBA (ng/ml)
4C	764.69±2	380.79±1.6
5C	-	923.04±0.9
6C	-	-
25K	-	640.78±2.6
26K	-	806.09±3.6

Siderophores are low molecular weight iron-chelating compounds secreted by bacteria, fungi, and plants under iron starvation, and make iron available for microbial and plant cells. Siderophores have a wide range of chemical structures, specific properties and the potentiality to bind a variety of metals in addition to iron. Siderophores act as bioremediators and chelation agents due to their heavy metal mobilization property, in addition to their important role in weathering, soil minerals and enhancing plant growth due to rhizobial uptake.

In the present study, results of the CAS agar test (Figure 21, Table 16) and $FeCl_3$ test (Table 16) revealed that almost all of the isolated soil microbes produced siderophores which help with plant growth and metal bioremediation.

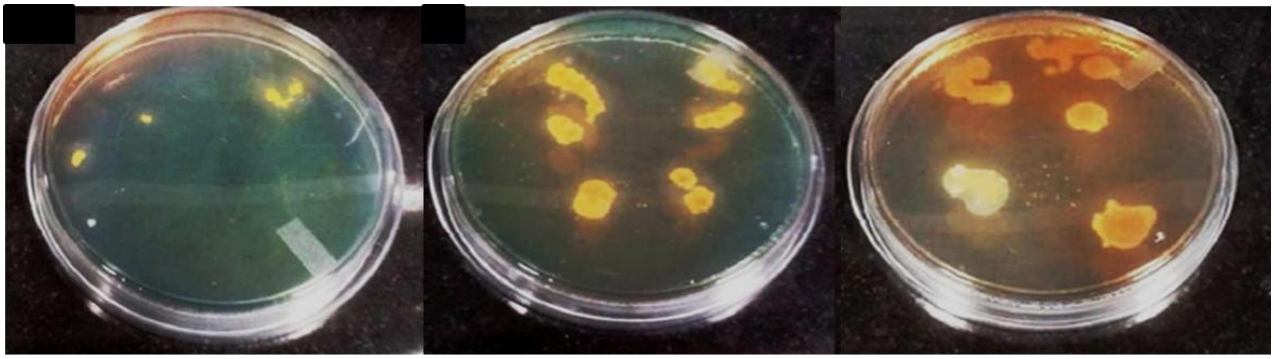


Figure 21: Detection of siderophore production on CAS agar plate after first, sixth and twelfth days of incubation. The disappearance of blue colour of CAS agar media and formation of orange pigment indicated that siderophore had produced because siderophore producing organisms produce orange depletion zones by extracting iron from the blue Fe-CAS complex.

Table 16. The results of the qualitative and quantitative assays for estimating siderophore production by the bacterial isolates:

Bacterial Strain	CAS Assay	$FeCl_3$ Test	Arnow's test for Catechol	Tetrazolium test for hydroxamate	Hydroxamate Siderophore (µg)/ protein(mg) (using Lowry method)
1-C	+++	+	-	+	0.03
2-C	+++	+	-	+	--
3-C	+++	+	-	+	0.03
4-C	+++	+	-	+	0.01
5-C	+++	+	-	+	0.01
6-C	+	+	-	+	0.03
7-C	+	+	-	+	--

8-C	+++	+	-	+	0.01
9-C	++	+	-	+	0.03
10-C	+++	+	-	+	--
11-C	+++	+	-	+	0.05
12-C (*Microbacterium radiodurans* K12016)	+++	+	-	+	0.01
1-E(*Bacillus xiamenensis* strain 1E0018)	-	+	-	+	0.03
2-E	+++	+	-	+	0.02
3-E	+++	+	-	+	--
4-E	+++	+	-	+	0.03
5-E	+++	+	-	+	0.03
6-E	+++	+	-	+	0.03
7-E	++	+	-	+	0.05
8-E	+++	+	-	+	0.03
9-E	+++	+	-	+	0.05
10-E	+++	+	-	+	0.01
11-E	+++	+	-	+	--
12-E	+++	+	-	+	0.05
13-E	+++	+	-	+	0.03
15-E	-	+	-	+	0.05
1-K	+++	+	-	+	0.01
2-K	+++	+	-	+	0.03
3-K	+++	+	-	+	--
4-K	+++	+	-	+	0.03
5-K	+	+	-	+	0.01
6-K	+++	+	-	+	0.02

7-K	+++	+	-	+	0.01
9-K	++	+	-	+	0.03
10-K	+++	+	-	+	0.01
11-K	+++	+	-	+	0.05
12-K	+++	+	-	+	0.03
13-K	+	+	-	+	--
16-K	+++	+	-	+	0.01
18-K	+++	+	-	+	0.05
25-K	+++	+	-	+	0.02
26-K (*Bacillus xiamenensis* strain 26K018)	+++	+	-	+	0.03

The role of rhizospheric microbe in decreasing plant heavy metal uptake from the surrounding environment:

The plants growing in the heavy metal contaminated regions absorb heavy metals from the rhizospheric soil, which subsequently reaches the human population several times magnified through the food chain, and is harmful to human health. Some previous studies revealed that the concentrations of heavy metals in dry fruit and other edible plant parts exceeded the allowed values approved by WHO and FAO [Neiem et al. 2009; Oueirolo et al. 2000; Husain et al. 1995]. The Bioconcentration Factor (BCF), is the ratio of the metal concentration measured in the vegetation to the concentration in the soil supporting that vegetation, can predict if the organism has accumulated the absorbed substance within their cell or not. A lesser than one BCF value in plants indicates that they probably only absorb, but do not accumulate heavy metals within the cell [Glick and Bashan, 1997]. On the other hand, greater than one BCF indicates that the metal is absorbed faster than it is removed and concentration builds up within the tissue i.e. bioaccumulation occurs. The BCF of some edible plants grown at the near vicinity of the sewage regions and consumed by local people,should be measured to investigate bioaccumulation and biomagnification of heavy metal through the food chain.

The average chromium content of edible plants collected from East Kolkata Wetland, Kestopur Khal, and Circular Canal was 751.92 ± 0.7 ppm, 16.08 ± 0.05 ppm, and 16.08 ± 0.05 ppm, respectively (Figure 23). This finding indicates that, in spite of the alarmingly high concentration of chromium in the soil (20864 ± 0.28 ppm, 187.1 ± 0.41 and 187.1 ± 0.15 ppm), the amount of soil chromium absorption by plant and the BCF value are quite low which may be due to the presence of rhizospheric soil microbes. The BCF (chromium) of the plants collected from East Kolkata Wetland, Circular Canal, and Kestopur Khal were 0.036±0.009, 0.028±0.01, and 0.085±0.01, respectively (Figure 24A).

The BCF of *Vigna radiata* plants grown in the presence of mixed microbial culture of East Kolkata Wetland, Circular Canal, Kestopur Khal and pure culture of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018, and the *Bacillus xiamenensis* 26K018 were 0.74±0.01, 0.58±0.09, 0.22±0.5, 0.88±0.01, 0.28±0.05, and 0.26±0.1, respectively. *Vigna radiata* grown in microbe free soils (control set) show a BCF value greater than one (1.05±0.02). These data indicate that the soil rhizospheric microbes play a critical role in controlling plant's chromium absorption in chromium-rich soil (Figure 24B).

The low chromium BCF values of the edible plants collected from the sewage regions indicate that these plants don't accumulate the absorbed soil chromium and therefore soil chromium is unable to enter food chain to cause bioaccumulation and biomagnification.

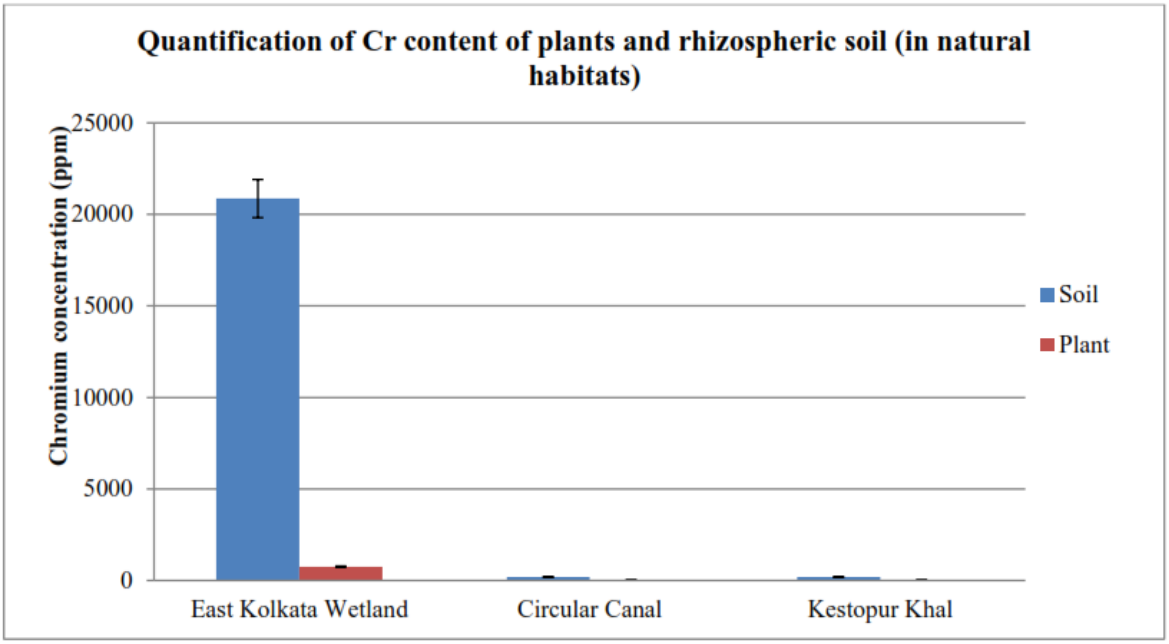


Figure 23: Quantification of the heavy metal content of the plants and soil collected from heavy metal contaminated rhizospheric soil

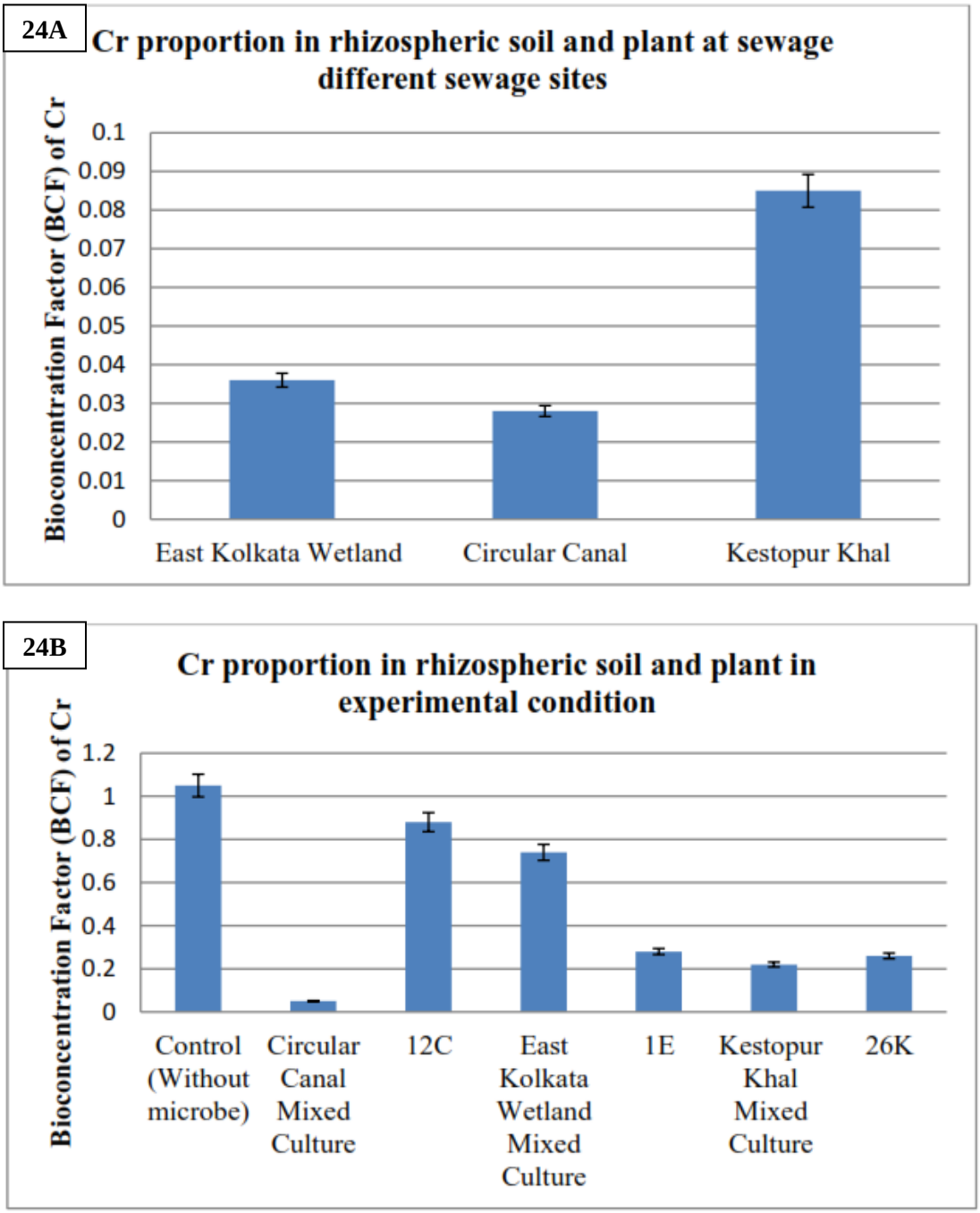


Figure 24: Chromium content [A] in sewage sites and [B] in experimental condition represented by the proportion of same in rhizospheric soil and plant

VI. Discussion

In order to understand the role of rhizospheric soil microbes in environmental heavy metal remediation, their community composition, mechanism in environmental heavy metal removal, and influence on the plants growing in the polluted soil were inspected.

The community composition study revealed that a lower amount of soil dehydrogenase enzyme producing aerobic bacteria grew at the different polluted sewage sites of Kolkata. Analysis of the amount of dissolved oxygen and chemical oxygen demand revealed that the sewage sites studied herein are highly polluted with organic materials, but have not yet crossed the borderline, which is why different pollutant tolerant plants, animals, and microbes are still able to grow and survive. Most of the Gram positive sewage microbes possess thickened cell walls and endospore. Endospore can withstand the presence of selective antibiotics and implement some strategies to overcome environmental stress.

Heavy metal resistance bacteria reduce environmental metal pollution by five main mechanisms (Inavena, 2009) as surface absorption, efflux, extracellular sequestration, intracellular sequestration and precipitation by oxidation or reduction of metal. The present study revealed that the chromium resistant microbes isolated from the heavily polluted wetland soil of Kolkata could accumulate the absorbed Cr(VI) within their cells and convert them to less hazardous Cr(III). Most of the isolated soil bacteria resist Co and Cd in addition to chromium. 16S rRNA based analysis exhibited that different species of *Microbacterium* and *Bacillus* were highly abundant in chromium contaminated regions. Sequence comparison study displayed that different geochemically important chromium tolerant and chromium resistant genes which translate numerous stress-induced proteins like ATP-dependent chaperon, ABC transporter, GTP binding protein, siderophore biosynthesis protein, chromium transporter protein, chromium reducing protein were native in *Microbacterium* [Learman *et al*, 2019] and some species of *Bacillus* [Wang *et al.* 2015, He *et al.* 2010]. Hemme *et al* (2016) reported that by lateral gene transfer, different sewage microbes become metal tolerant permanently.

Soil microbes can bind heavy metals in the soil by producing siderophores [Glick and Bashan 1997; Braud et al. 2009a; Rajkumar et al. 2010] and microbial acids [Rózycki and Strzelczyk 1986; Renella et al. 2004]. Most of the soil microbes investigated here produced considerable quantities of hydroxamate siderophores and organic acids, evaluated by the positive results in the

MRVP test, which have a significant impact on soil cation mobility and the regulation of nutrient uptake by the plants, thus indicating that these microbes can reduce the absorption of heavy metals by plants by producing siderophores and extracellular acids. Additionally, the microbial isolates were found to produce the plant growth promoting hormone, IBA and IAA. Previous reports have indicated that IAA produced by rhizospheric microbes, alters the endogenous IAA pool in plants [Arshad et al. 2007; Glick 2012] and increases the length and surface area of plant roots [Glick 2012].

The results reveal that morphologically and biochemically identical soil microbes, residing in the different sewage regions of Kolkata, exhibit a similar trend in chromium removal and soil-plant interaction.

VII. Conclusion:

It is possible to conclude that isolated strains can serve as potent bioremediating bacteria, because of their significantly higher chromium tolerance, reduction and accumulation ability. Heavy metal profiling of the soil samples and the plants growing in the sewage soil indicated that the resident soil microbes greatly influence heavy metal absorption by the plants. From these findings, it may be concluded that, the isolated chromium removing, eco-friendly strains have a great impact on agro-economic society as they could be applied to eliminate environmental chromium, as well as improve plant growth in heavy metal contaminated, poorly cultivable soil and decrease the risk of metal accumulation and biomagnification by the edible plants that are consumed regularly. Further field application should be required to clarify their bioremediation and plant interaction strategies.

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IX. Plagiarism certificate:

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XI. Additional Documents:

a. Certificate of conferences attended

ICEES 2011

3rd INTERNATIONAL CONFERENCE ON ECOTOXICOLOGY & ENVIRONMENTAL SCIENCES
(ICEES-2011) November 28-30, 2011

Organized by
INSTITUTE OF ECOTOXICOLOGY AND ENVIRONMENTAL SCIENCES
Y/7, XL Purbachal, Salt Lake, Kolkata-700097, INDIA
Website: http://www.iees.org.in

Certificate of Participation

This is to certify that Dr/Mr/Mrs/Ms. Chitrita Chatterjee
Attended/presented a paper entitled "Isolation and partial characterization
Bacterial ... at the ICEES-2011 held during
28-30 November, 2011 at the Mizamar Residency, Panji, Goa-403001, INDIA.

Prof. Shyamal Majumdar
Chairman, International Scientific Committee, ICEES-2011
&
Professor Emeritus of Biology, Biology Department, Lafayette College, Easton, PA, USA
Editor Pennsylvania Academy of Science, Journal & Books

Dr. B. Bhattacharya
Chairman, Organizing Committee, ICEES-2011
&
President, IEA & IEI, Kolkata, INDIA

Certificate of Participation

This is to certify that Chitrita Chatterjee, Presidency University has participated and presented a poster entitled "The role of heavy metal resistance Indole producing Municipal sewage microbes in plant heavy metal absorption and accumulation" in the "6th India Biodiversity Meet 2019 (International Conference)", held at Indian Statistical Institute, Kolkata, from 14th -16th February, 2019.

Abhishek Mukherjee
Organizing Secretary

Debjyoti Bhattacharya
Head, ASRU, ISI

6th INDIA BIODIVERSITY MEET 2019

10th Year Celebration of Excellence in Science at IISER Kolkata
"Advances in Life Sciences"

Certificate
OF PARTICIPATION

This is to certify that Ms. Chitrita Chatterjee has presented a poster in the 10th Year Celebration of Excellence in Science at IISER Kolkata, "Advances in Life Sciences" organised by Indian Institute of Science Education and Research Kolkata, during January 13 - 15, 2017.

Convenor

Bijoy Krishna Girls' College,Howrah
5/3 M.G. Road, Howrah - 711 101, West Bengal,India
www.bkgc.org

UGC-CPE sponsored International Conference on
Facets of Basic Sciences & Applications (FBSA)

Organised by IQAC & Departments of
Botany, Chemistry, Computer Science, Electronics, Mathematics,Micro-Biology, Physics, Statistics and Zoology

Certificate of Participation

Certified that
Ms. Chitrita Chatterjee
of
Presidency University, Kolkata
has attended and presented a paper entitled
"ISOLATION, CHARACTERIZATION AND GENETIC STUDY OF HEAVY METAL RESISTANCE SEWAGE BACTERIA FROM TOLLY NULLAH AND THEIR POTENTIALITY IN PLANT GROWTH PROMOTION"
in the Conference held on February 5 - 7, 2018

Convenor FBSA 2018 | Organising Secretary FBSA 2018 | Coordinator IQAC | Principal BKGC & Conference Chair FBSA 2018

"The Green Planet: past, present and future"

Prof. / Dr. / Mr. / Ms. Chitrita Chatterjee of Department of Life Sciences, Presidency University has attended and delivered an invited lecture/chaired a session/ co-chaired a session/presented a paper/ a poster entitled Investigation of plant growth and biomass reward from Tolly nullah in the International Conference on "The Green Planet: past, present and future" from 21st to 23rd December, 2016 held at CAS-VII, Department of Botany, University of Calcutta, Kolkata, India.

Dr. V. Singh (Convener) | Prof. S. Bera (Convener) | Prof. A. Acharya (Convener) | Prof. P. Pal (Convener & HoD)

National Conference on
Future India: Science and Technology
27th & 28th February, 2019

This is to certify that Ms. Chitrita Chatterjee of Presidency University participated & presented a paper in the National Conference on "Future India: Science and Technology" on 27th and 28th February, 2019 held at City College, Kolkata - 700 009.

Prof. Tushar Kanti Ghosh, Convener, IEM, Kolkata Chapter | Dr. S. K. Ghosh & Dr. S. K. Ghosh, Organising Secretary | Dr. S. K. Chattopadhyay, Principal, City College, Kolkata

CERTIFICATE OF ATTENDANCE

DBT-BUILDER Sponsored National Symposium, February 19-20, 2015

"Environmental Impact on Biodiversity and Plant Development"

Certified that

CHITRITA CHATTERJEE

PRESIDENCY UNIVERSITY

has attended the National Symposium organized by the Department of Biological Sciences and presented an invited talk/poster

PRESIDENCY UNIVERSITY KOLKATA

Dr. Malay Das, Convener & Organizing Secretary

Dr. Madhumita Chowdhury, Head of the Department

Dr. Kamala Gupta, Treasurer

National Symposium on

Evolving Plant Biology: From Chromosomes to Genomics

Certificate of Participation

This is to certify that Mr./Ms. Chitrita Chatterjee, Presidency University, Kolkata has participated in the National symposium on Evolving Plant Biology: From Chromosomes to Genomics held at Bose Institute and The Ramakrishna Mission Institute of Culture, Kolkata

INSA AWARD LECTURES & PANEL DISCUSSION ON "BIOMOLECULAR RESEARCH AND INFORMATICS: FUTURE IN INDIA"

ORGANISED BY PRESIDENCY UNIVERSITY, KOLKATA & DBT CENTRE FOR BIOINFORMATICS

27TH FEBRUARY 2012

This is to certify that Dr. / Mr. / Ms. CHITRITA CHATTERJEE of PRESIDENCY UNIVERSITY attended the INSA AWARD LECTURES AND PANEL DISCUSSION ON "Biomolecular Research and Informatics: Future in India" held on the 27th of February 2012 at Presidency University, Kolkata.

PROFESSOR MALABIKA SARKAR, VICE-CHANCELLOR, PRESIDENCY UNIVERSITY & CHAIRPERSON, DBT CENTRE FOR BIOINFORMATICS, P.U.

DR. ARNAB SAHA, COORDINATOR, DBT CENTRE FOR BIOINFORMATICS, P.U.

IISER PUNE

35th Annual Meeting of PTCA (India) & National Symposium on Advances in Plant Molecular Biology & Biotechnology

March 10 – 12, 2014

Certificate

This is to certify that Chitrita Chatterjee has attended the 35th Annual Meeting of PTCA (India) & National Symposium on Advances in Plant Molecular Biology & Biotechnology, organized by Indian Institute of Science Education and Research (IISER), Pune.

Dr. Anjan K. Banerjee

PTCA

b. First page of published manuscript

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Characterize heavy metal tolerant rhizospheric sewage bacteria isolated from Tolly nullah (with special emphasis on strain *Microbacterium radiodurans* K12016)

Chitrita Chatterjee[•]
Rabindranath Bhattacharyya[••]
Shampa Sarkar (Biswas)[•••]

Abstract

Because of rapid industrialization and urbanization, heavy metals are deposited in the environment in frightening quantity. Literature survey manifested, that the heavy metal contaminated soil born microbes can be a potent mode of environmental heavy metal degradation. In the present study, to reveal microbial heavy metal tolerant activity rhizosperic microbes were isolated from sewage sludge of circular canal (Tolly Nullah) and characterize. Microbial heavy metal resistance pattern was checked against four heavy metal salts (Cr_2O_3, CrO_3, $CdCl_2$ and $CoCl_2$) individually and in consortium. Plant growth promoting rhizobacterial (PGPR) traits of isolated microbes were evaluated. One interesting isolated strain (*Microbacterium radiodurans* strain K12016), identified by 16s rDNA, shown total tolerance against 100 mM Cr_2O_3 (Cr^{3+}) and also mentionable Cobalt and hexavalent Chromium (Cr^{6+}) tolerance, but didn't produce a significant amount of plant growth promoting IAA whereas the most of the rest isolates produced adequate amount of IAA. 16s rDNA of that particular isolate was analyzed and phylogenic tree was constructed using the neighbor-joining method to ensure its taxonomic position. Furthermore experimentation and gene study should require to determining heavy metal resistance mechanism of *Microbacterium radiodurans* K12016.

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Keywords:

Heavy Metal tolerance;
Biochemical analysis;
Antibiotic resistance;
Indole Acetic Acid (IAA);
16s rDNA.

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1. Introduction

As a result of different unrestricted anthropogenic activities, different concerning heavy metals like Chromium (Cr), Lead (Pb), Cadmium (Cd), Cobalt (Co), Nickel (Ni), Iron (Fe) are alarmingly increased in the environment, particularly the areas where waste materials from different industrial sectors, tannery,

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